The impact of storage facilities on animal feed quality with reference to mycotoxin contamination around Ngaka Modiri Molema District, North West Province

K G Setsetse

orcid.org/ 0000-0002-4631-9258

Dissertation accepted in fulfilment of the requirements for the degree of Master of Science in Agriculture in Animal Health at the North West University

Supervisor: Prof Mulunda Mwanza

Graduation ceremony April 2019

Student number: 22538364

DECLARATION

I, KGOMOTSO GALIAN SETSETSE declare that the dissertation entitled "The impact of storage facilities on animal feed quality with reference to mycotoxin contamination around Ngaka Modiri Molema District, North West Province", hereby accepted in fulfilment of the requirements for the degree of Master of Science in Agriculture in Animal Health at the North-West University, is the study done by me and it has never been published or done elsewhere before. I further declare that all sources cited are indicated and acknowledged by means of an inclusive list of references.

.....

Signature

Name: Kgomotso Galian Setsetse

Student number: 22538364

ABSTRACT

The improper storage system of feed is a major factor influencing the presence of fungi and mycotoxin contamination. Hence the aim of the study was mainly to compare the impact of storage facilities on fungal and mycotoxin contaminants in animal feed collected from emerging farmers' and commercial supplier's storages in the Ngaka Modiri Molema District, North West Province of South Africa. To achieve this, a survey and an interview were carried out during the collection of samples to obtain the views and level of knowledge of farmers and suppliers in connection with the manner in which they stored animal feed. It was noted that major challenges faced by emerging farmers versus feed commercial suppliers were that they were not knowledgeable about proper feed storage, effects of mycotoxin contamination on feed and, not educationally trained. It was also found in this study that participated farmers mainly used two types of storage systems, about 41.7% used open storage system and 58.3% used closed storage systems and their animal feeds were preserved in bags or dustbin, whilst feed commercial suppliers mainly used closed storage. Data collected also revealed that majority of farmers did not produce their own feed, fed to their animals but purchased from different feed suppliers around their individual areas. Feed contaminant, in this case, could have been from different sources such as the field obtained from the processing, the supplier's storage or recipient farmer's storage. Contaminants could persists in harvested and stored grain and grow in storage when moisture content becomes favourable. This may explain their presence in these analysed samples from both storages of emerging farmers and feed suppliers storage by late harvesting and proper storage. There were a 100 samples of which 40 were collected from closed and open storages from emerging farmers and 60 samples from commercial supplier's closed storages. The moisture content was determined using the oven drying method, and fungal isolation and identification were performed using serial dilution and cultured on malt extract agar (MEA), potato dextrose agar (PDA), and Sabouraud dextrose agar (SDA) media. Isolated fungi were confirmed using the molecular techniques and Polymerase Chain Reaction (PCR). The mycotoxins extraction, determination, and quantification were done using the ELISA and HPLC and TLC methods. The results obtained revealed that emerging farmers, in general, did not have knowledge of fungi and mycotoxins as well as the impact of storage on the quality of animal feed. Whilst suppliers were knowledgeable about fungi and mycotoxins but did not implement necessary measures to keep the feed in a proper environment. Data obtained from the sample analysis showed significant differences ($P>0.05$) in moisture contents

among the feed types. Silage samples had the highest moisture content as compared to other types of feed. Results for fungal isolation showed that the fungal loads (Cfu/g) in cultures from the feed samples collected from feed suppliers' closed storage were significantly higher than the ones from the open as well as the closed storages from the emerging farmers. In addition, the fungal analysis revealed that 78% of the screened samples were contaminated with fungi, of these fungi, the most important mycotoxin-producing strains were *Aspergillus* spp (42%), *Penicillium* spp (26%) and *Fusarium* spp (10%). Among isolated fungal strains, *A. flavus, A. oryzae, A. terreus, A. fumigatus, A. clavatus, A. niger, A. parasisticus, A. nomius, P. verrucosum, P. chrysogenum, P. polonicum, P. rubens, P. brevicompactum* and *F. oxysporum* were the main contaminants. The study also found that there was a statistically significant difference across storage systems ($P>0.05$), with samples obtained from the supplier's closed storages being more contaminated than the closed and open storages of emerging farmers. Whilst samples from among the farmer's storage samples collected from the closed tanks were more contaminated than those of open storage ($P>0.05$). The results obtained revealed that Aflatoxin (B1, B2, G1, and G2), were the predominant mycotoxins amongst all the contaminants with about 97.7% occurring in emerging farm storage (open & closed) and commercial feed suppliers storage 100% with a mean concentration of 326.3 ppb and 422.4 ppb respectively. Emerging farms and commercial feed supplier's samples respectively were contaminated with Ochratoxin A with a mean concentration of 387 ppb and 575 ppb respectively. Zearalenone mean concentrations were 31.3 ppb on emerging farm storages and 7.32 ppb from commercial feed supplier's storage with respective contamination of 8.3% and 23.3% while fumonisin (B_1, B_2) in emerging farms and commercial supplier's samples had a mean value of 525.5 ppb and 193.67 ppb, respectively.

The study clearly showed that both closed and open storages had fungal and mycotoxin contamination. Although the closed storages showed high contamination with fungi and mycotoxins, the study noted that this was due to improper control of the environment in the storage. The open storage has a major challenge that there are no means of controlling the environment during storage. Feed quality regarding fungi and mycotoxin remain primarily a training issue for farmers so they can be able to control the storage and reduce the risk of contamination. Therefore environmental control is the key to fungal and mycotoxin control. Storage duration, type of feed and type of storage have a significant influence on fungal growth and mycotoxin production.

DEDICATION

I dedicate this dissertation first and foremost to our heavenly father for he always rises me above all odds, this is by far my biggest achievement.

My guardian angel Dikeledi Elisabeth Setsetse (1960 – 2014), Thank you for everything. I am the person I am today because of everything you have done for me with love and nurturing. I hope wherever you are, you are proud of your little girl. This one is for you.

My daughter Bokamoso Vuyelwa Setsetse, you're my daily inspiration. I thank God, every day for choosing me to be your mom, I am doing all this for you.

"*For I know the plan I have for you, plans to prosper you not to harm you, plans to give you a hope and future "– Jeremiah 29:11*

ACKNOWLEDGMENT

Firstly, I would like to thank the almighty God our father for giving me the wisdom and strength to work towards completion of this qualification, continuing to pass my greatest gratitude to:

Prof. Mulunda Mwanza my supervisor, for his supervision and patience in allowing me to push hard and manage to finish my work.

Dr. Lubanza Ngoma for his assistance in the laboratory with regards to Molecular analysis.

Mr. Ngwane, for his patience, when assisting me with my laboratory analysis, and pushed me to work harder.

Dr. Modupeade Adetunji for her mentoring and helping me with my writing, and not just only that, and for her words of encouragement.

My fellow students and the large animal team, especially Mr. Mjekula, with their assistance at collection of samples during outreach trips organized by the Animal health department. Thank you for the cooperation of the suppliers and farmers in the rural areas of the district of Ngaka Modiri Molema District in the North-West Province, who were interviewed. This study would not have been possible if it were not for your co-operation.

Dr. Volition Montshiwa for his assistance in helping me to analyze my data statistically.

I am also grateful for the financial support granted by the North-West University (NWU) postgraduate bursary, HWSETA and the National Research Fund (NRF), thank you very much for making it possible to achieve this.

My family, daughter, and friends, I thank you for your unconditional support throughout the difficulty of the course study, for not losing faith in me when things looked shallow. I would not have reached this far if it were not for you all. Lastly but not least, I would like to give all the credit to my late mom (Dikeledi Elisabeth Setsetse). Thank you very much, I am what I am today because of you. I know you are looking down at me and very proud. This is for you.

DEFINITIONS

Aflatoxin: A complex of four mycotoxins produced by *Aspergillus flavus*; often found in peanut products.

Carcinogenic: Ability of a substance to cause cancer when administered to an organism.

Emerging farmers: Are referred as a group of smallholder farmers, who were previously excluded from the mainstream of the economy in South Africa.

Feeds: food for reared animals

Fungi: A eukaryotic single-celled or multinucleate organisms that live by decomposing and absorbing the organic material in which they grow, comprising the mushrooms, moulds, mildews, smuts, rusts, and yeasts.

Grain: Single, the dry indehiscent fruit of a single seed that is fused to the ovary wall.

Hepatocarcinogenic: Ability of a substance to cause cancer of the liver when administered to an organism.

Hyphae**:** Microscopic threads that make up the body of most fungi.

Leukoencephalomalacia: Refers to the neurotoxic disease of horses.

Mould: A fungus that grows in the form of multicellular filaments called hyphae.

Mycosis: Refers to the generalized invasion of living tissue by growing fungi.

Mycotoxicosis: Used to describe the action of mycotoxins and is frequently mediated through several organs notably the liver, kidney, lungs and the nervous, endocrine and immune system.

Mycotoxin: A natural toxin of fungal origin.

Secondary metabolites: Organic compounds produced by an organism which are not directly involved in normal growth, development, or reproduction of the organism.

Teratogenic: Ability of a substance to cause abnormalities in the embryo or fetus when administered to the maternal organism.

Toxin: A poisonous substance

LIST OF ABBREVIATIONS

AF: Aflatoxin

CFS: Commercial feed supplier

DON: Deoxynivalenol

EF: Emerging farms

ELISA: Enzyme-Linked Immunosorbent Assay

FAO: Food and Agricultural Organization

HPLC: High-Performance Liquid Chromatography

IAC: Immuno-Affinity Column

MEA: Malt Dextrose Agar

MRL: Maximum Residue Limits

Nm: Nanometer (nm)

NMMD: Ngaka Modiri Molema District

OTA: Ochratoxin A.

PCR: Polymerase Chain Reaction

PDA: Potato Dextrose Agar

RF: Retention factor (R_F)

SDA: Sabouraud Dextrose Agar

Spp: Species

TLC: Thin Layer Chromatography

WHO: World Health Organization

ZEA: Zearalenone

LIST OF UNITS

°C:	Degree Celsius
µg/ml:	Microgram per milliliter
%:	Percentage
Cfu/g	Colony forming unit/gram
µg/kg:	Microgram per kilogram
µg/g:	Microgram per gram
µL:	Microliter
G:	Gram
Hrs:	Hours
Kg:	Kilogram
L:	Litre
Mins:	Minutes
Na/g	Nanogram
Nm:	Nanometre
Ppm:	Parts per million
Ppb:	Parts per billion
S:	Second
V:	Volume
V/v:	Volume/volume

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

GENERAL INTRODUTION

Mycotoxins are "low-molecular-weight natural products produced as secondary metabolites by filamentous fungi that consist of a toxin and chemical of heterogeneous assemblage that is assembled together only because the members can cause illness and death in human beings and other vertebrates" (Zain, 2011). Contamination of food and agricultural commodities by various types of toxigenic moulds is a serious and widely neglected problem, regardless of decades of extensive research, mould infection still remains a challenging problem (Munkvold, 2003a). Moulds that produce mycotoxins, grow in favorable condition and may contaminate human food and animal feed prior to and during yield periods or improper storage (Fink-Grernmels, 1999).

Generally, numerous fungi are toxigenic and not all secondary metabolites from fungi are toxic, even though some of the mycotoxins are formed by more than one fungal specie, hence more than one mycotoxins can be detected on a contaminated feed-stuff.

Many fungi of the genera *Fusarium, Alternaria, Penicillium, Aspergillus, Cladosporium*, and others are well well-known producers of over 500 known mycotoxins, but that of major public health and agro-economic importance includes aflatoxins (AF), Ochratoxin (OT). Trichothecenes (T), zearalenone (ZEN), fumonisins (F), tremorgenic toxins, and ergot alkaloids (Stojanovic *et al*, 2005).

Animal feeds are some of the most susceptible and essential commodities that are likely to get contaminated with mycotoxins and this forms part of the farm animals-to-human food chain; therefore, infectious and non-infectious hazards present in animal feeds pose a threat to human health. This may result in economic loss and transmission of toxins into the food chain (da Rocha *et al.*, 2014). The importance of the health risk of these contaminants can be determined by the amount of mycotoxin ingested and the level of exposure which can either be through contact with contaminated materials or inhalation and absorption through the skin (Bankole & Adebanjo, 2003). Factors contributing to the presence or production of mycotoxins in food or feeds include poor storage, environmental and ecological conditions and many times most factors are beyond human control (Hussein & Brasel, 2001). Therefore, the aim of the study was to assess the possible correlations between the types of storage facilities, fungal occurrence and the associated mycotoxins in animal feeds collected from emerging farmers and commercial supplier's storage facilities around the Ngaka Modiri Molema District, North West Province of South Africa.

1.2 Problem identification

Numerous studies have been done generally regarding mycotoxins, but not much has been done to determine the right storage facility for emerging farmers on how to store their livestock feed to avoid growth of fungi. Animal feed plays an important part in the food chain and has implications for the composition and quality of the livestock products that people consume (Kan & Meijer, 2007). Furthermore, most emerging farmers around South Africa, generally store their feeds under the roof of the farmer's houses or on the floor in garages and commercial farms store their feeds in silos or in bags.

The impact of these storage models and environmental conditions affect the quality of feed, particularly in areas or zones that promote fungal infection and subsequent production of mycotoxins contamination (Mwanza, 2012).

There are two important mycotoxigenic fungi that are associated with stored feed, these are *Aspergillus flavus* and *Fusarium verticillioides* (Krnjaja *et al.*, 2013).

These mycotoxins pose a risk to animal health and can affect livestock production for several species, but the risk to public health is considered low; in all cases, the feed of animal origin only contributes marginally to the total human exposure to these toxins (Zachariasova *et al.*, 2014). Fumonisins are q mycotoxins (Gelderblom *et al.*, 1988), that can cause fatal diseases in horses (Leukoencephalomalacia) and swine, possess cancer-promoting activity in rats, and are associated with porcine pulmonary edema (Norred & Voss, 1994). In addition, oesophageal cancer in humans has been related to consumption of maize with high concentrations of fumonisins (Nelson *et al.*, 1994).

Therefore it is the "responsibility of the researchers, feed business operators, department of agriculture and farmers to ensure that feed placed on the market or fed to any food-producing animal is safe, has no adverse effect on human or animal health and therefore meets the guidance values for mycotoxins" (Verstraete, 2008).

1.3 Importance of the study

This study will serve to inform farmers on effect of storage on toxin production and current toxin levels observed. In addition, it will assist in training farmers on how to correctly store their feed to avoid contamination of any form. Farmers and animal feed suppliers should consider the following when storing their livestock feedstuff; the type of storage structure, hygiene, avoid insect infestation because of it an enormous effect of causing fungal infection and mycotoxin contamination (Fandohan *et al.*, 2006).

A questionnaire disclosed information about how farmers store their animal feeds, and further laboratory analysis on the feed that was carried out on the collected sample to evaluate whether feed bought by farmers are contaminated from the suppliers or contaminated during the storage by emerging farms.

1.4 Therefore objectives of this research were:

- To assess the effects of these storage facility type on fungal contamination and mycotoxins accumulation in animal feed collected from farmers and commercial feed suppliers
- To determine the point of mycotoxins contamination in the feed from the farmers' store to the supplier's store.
- To quantify the fungi and mycotoxin contamination in animal feed in different storage conditions in the Ngaka Modiri Molema District.

CHAPTER II

LITERATURE REVIEW

The occurrence of mycotoxins in feedstuff is not only a problem in developing countries, in fact, it affects agribusiness in many countries across the globe, influencing even impeding exportation, reducing livestock and crop farming production and, in some countries, affecting human health (Fandohan *et al.*, 2006). The intake of mycotoxins by humans occurs mainly through eating contaminated plant products, as well as through products derived from foods such as milk, cheese, meat and other animal products (Milićević *et al.*, 2010).

A wide range of agricultural commodities can be contaminated with mycotoxins from production in the fields, during harvest, transportation or in storage (Méndez & Moreno, 2009) as shown in Figure 2.1. Mycotoxins can enter into the human and animal food chains through direct or indirect contamination, therefore the indirect contamination is through ingredients from feedstuffs which were before contaminated by a toxigenic fungus, and even though the fungus may have been eliminated during the processing and the mycotoxins remain in the final product. Whilst, direct contamination, on the other hand, occurs when food or feed becomes infected by a toxigenic fungus, with the subsequent formation of mycotoxins (Bohra & Purohit, 2003).

A lot of the product deteriorations are caused by storage moulds which results in a decrease of germination ability, loss in grain weight, discoloration of feeds and mustiness, chemical and nutritional changes, and mycotoxin contamination (Malaker *et al.*, 2008). Diseases caused by mycotoxins are called mycotoxicosis, the condition can either be acute or chronic or both depending on the kind of toxins and dose (Richard, 2007). In animals, acute diseases include liver and kidney damage, attack on the central nervous system (CNS), skin diseases and hormonal effects. Among the mycotoxins, aflatoxins produced by *A. flavus, A. parasiticus, A. nomius* and the most potent natural carcinogenic compound causing mutation (Choudhary & Kumari, 2010). Reliable calculations show that approximately 25%-50% of all the commodities produced globally, especially basic foodstuffs are contaminated in some way with mycotoxins (da Rocha *et al.*, 2014).

Therefore, the safety of food and feed for humans and animals consumption is of the highest priority with regards to the regulations of agricultural and food industries. This is particularly significant in the markets which are compromised by the sale of low quality or harmful food caused by mycotoxins. ‘Most developed counties will not permit the importation of commodities containing amounts of mycotoxins above specified limits’ (Malaker *et al.* 2008).

On the contrary with regards to livestock feeds, mycotoxins pose the greatest threat as the practices that reduce mycotoxin contamination may differ depending on climate region and the type of the crop (Bryden, 2012).

Mycotoxigenic fungal growth can arise in storage because of moisture variability within the grain itself or due to moisture migration resulting into cooling of grains located near the interface with the wall of the storage container/silo (Kabak *et al.*, 2006). Hence, it is important to control aeration and periodical monitoring of the moisture content of silos, adequately because it plays a major role in restriction of mycotoxin contamination during storage period (Kabak *et al.*, 2006). The moisture levels in stored crops are some of the most critical factors in the growth of mycotoxigenic moulds and in mycotoxins production. These levels are some of the main reasons for mycotoxins problems in grain produced in developing countries (Magan *et al.*, 2011).

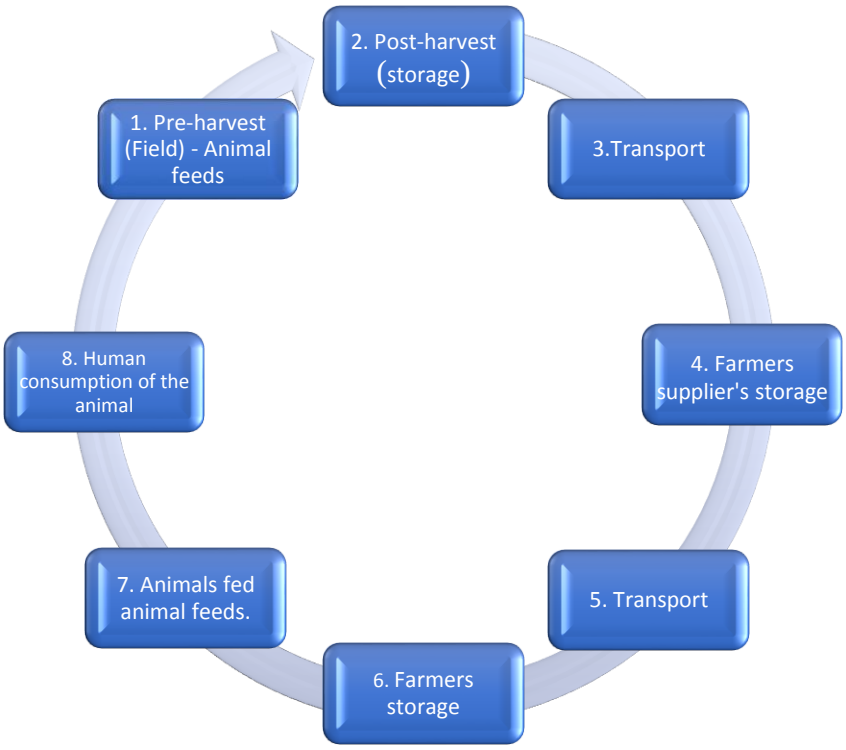


Figure 2.1: The pathway of animal feed storage and consumption (Hsieh, 1990)

2.1 Emerging farmers in Ngaka Modiri Molema District, South Africa.

Ngaka Modiri Molema District Municipality is a predominantly rural region where agricultural farming and livestock form the economic backbone of the district. The district is one of the four district of the North West province in South Africa. Its temperature ranges between 17° to 31°C during summer and 3° to 21°C in the winter seasons. Total annual rainfall is about 360mm during summer months, between October and April (Botlhoko & Oladele, 2013). The North West produces 18% of South Africa's total maize, a crop whose yields have been shown to be highly sensitive to rainfall changes (Blignaut *et al.*, 2009). In particular, small-scale farmers and emerging farmers in the North West province are likely to experience revenue losses if rainfall decreases markedly (Benhin, 2006). Emerging farmers in South Africa are considered to be classified as smallholder farmers, who were previously excluded from the mainstream, hence it is difficult to define emerging farmers (Ortmann, 2003).

Figure 2.2: Classification of South Africa's farming sector (Pienaar *et al*., 2013)

2.2 Animal Feeds

Animal feeds are food given to domestic animals, which has two basic types; namely fodder and forage (Wiktionary, 2016) which includes hay, straw, silage, compressed and pelleted feeds, mixed rations, sprouted grains and legumes (Fageria *et al.*, 2010). Animal feeds such as maize, cottonseed, and greens are most likely to be affected by mycotoxins (Diekman & Green, 1992). The amount of grain used to produce the same unit of meat varies substantially. According to an estimate reported by the BBC in 2008, 'Cows and sheep need 8 kg of grain for every 1kg of meat they produce pigs about 4 kg and the most efficient poultry units need a mere 1.6 kg of feed to produce 1 kg of chicken (BBC news, 2008). However, the occurrence of mycotoxin remains a threat in developing countries, in fact, it affects agribusiness in many countries, reducing livestock and crop farming production with increased mortality and morbidity in animals as well as in humans.

2.2.1 Types of animal feed

Roughage

Roughage is referred to as forage this is of the type of plant material which consists of high fibre and relatively low in digestible nutrients eaten by grazing livestock, although many mycotoxins have been reported to have been found to occur in forages either on the field or in storage (Roberts *et al.*, 2005).

Concentrates

Concentrates are usually kept in a barn or enclosed shed, this is ensure that they are kept dry and free of moulds. They take up less storage space and considered to be less of a fire hazard. Feed kept outside or exposed to moisture can develop mould quite quickly (Wiktionary, 2016)

Silage

Silage is a type of feedstuff that is made from grass, maize, sorghum and any other green plant, it is not just grain that is stored in high moisture for processing of under anaerobic condition for fermentation (Coblentz, 2006). Although the process leads to the formation of mould just as *Aspergillus* genera, which are mostly reported to be the mould contaminant silage which caused mycosis and induced abortion in pregnant cows (Richard *et al.*, 2009).

Hay

Hay is known as a combination of fodder and forage product plants used mainly for grazing of grazing ruminants. Hay is often stored under open an open shed and kept covered, and commonly packed in bales that usually increase the chances of mould invasion (Fink-Gremmels, 2008). Generally, about 15% moisture levels have been reported to be found in hay and the presence of *Aspergillus fumigatus* (Shadmi *et al.*, 1974).

Legume

Legumes are primarily grown agriculturally as grain seed called pulse which is reported to be the second largest group of forage which includes alfalfa, clover, soybeans, and peanuts that are consumed by ruminants (Fink-Gremmels, 2008). The fungus called *Rhizoctonia leguminicola* is predominant in legume leaves causing slobber disease in dairy cattle (Daya-Khilosia, 2011).

Lucerne

Lucerne is a digestible fibre and contains high protein source used to feed animals. It is used for grazing, hay, and silage, as well as a green manure and cover crop (Khilosia, 2011). If left too dry, it reduces the nutrient level. Reports on animal feed showed that *Aspergillus flavus* has been isolated from Lucerne (Ghiasian & Maghsood, 2011).

Soya bean

Soya bean is the richest plant source of protein, the only plant source that contains all eight essential amino acid, widely grown for its edible bean which has numerous uses (Boland *et al.*, 2013). Soya beans have been reported to have the presence of Aflatoxin B1 from *Aspergillus flavus* (Bandyopadhyay *et al.*, 2007).

Maize

Maize is considered a large grain extensively cultivated as a cereal crop on earth. It is a staple crop, and human rely on it as a primary source of nutrition, and it also used as a livestock fodder (Gorman & Kang, 1991).

Cottonseed

Cottonseed contains a rich source of protein, energy and provides the basis for textile fibers that remain as a valuable raw material for a variety of foodstuffs and feed, which was reported to be imported to South Africa during 2004 (Crossan *et al.*, 2006). Therefore most of the cottonseed used in South Africa has been reported to have a non-aflatoxigenic seed, which must be stored adequately in a ventilated area to avoid spoilage and growth of fungi (Crossan *et al.*, 2006).

Barley

Barley is commonly used as animal fodder and for the production of silage, as well as a source of fermentable material for certain beverages (e.g. beer) and as a component of different food. Many studies have suggested an increase in consumption of barley in feed and food to decrease heart disease and overall mortality (Newton *et al.*, 2011).

Wheat

Wheat is almost certainly the most common cereal available all over the world and it is even in higher demand in recent years due to its sample health benefits, although it has been reported that notorious mycotoxin producers such as aflatoxigenic fungi were isolated from it during analysis of feedstuff (Turner *et al.*, 2012).

Sunflower seed

Sunflower seed is a source of essential fatty acids, vitamins, and minerals which provide about 40% of the oil cake that is processed for animal feed. Correspondingly studies that show that about 20% seed of moisture content and regulated storage prevent sunflower from being germinated with mycotoxin (Nawaz *et al.*, 1997).

Molasses

Molasses is dark sticky by-product of processing sugar cane into sugar, it is considered to be a source of energy and minerals. It contains significant qualities of minerals such as copper, zinc, iron and manganese. It increases feed intake and improve palatability in animals (Daly-Koziel & Walters, 2012).

Salt

Salt supplementation is a critical part of a nutritionally balanced diet for animals. It is used as a required supplement to composed of both sodium (Na) and chlorine (Cl). Severe deficiency of sodium and chloride leads to cerebral edema, seizures, coma,brain damage and death (Cardon *et al.*, 1951).

Pelleted compound feed

Pelleted feeds are agglomerated feeds formed by extruding individual ingredients such as starch, fiber, protein, and lipids. The purpose of pelleting is to take a finely divided, dusty, unpalatable and difficult to handle feed material, moisture and pressure changing its form into larger particles. All livestock feeders agree that animals make better grains on pellet feed than a meal ration hence this processing also helps in monitoring mycotoxin contamination of the product as well (Sudekum *et al.*, 2008).

Total mixed rations

The total mixed ration is a method of feeding animals especially cows, combining all forages, grains, protein feeds, minerals, vitamins an feeds additives formulated to a specified nutrient concentration into a single feed mix. This method is mainly used to balance rations that are consumed by cows, for energy and mainitaining their physical characteristics, which we now refer to as feed particles size, required for proper rumen function (Tyasi *et al*., 2003).

2.3 Feed storage conditions

Generally, in most emerging farms in South Africa, they keep their feed either in a vehicle garage, under the roofs of the farmers 'houses, or on the floor in the houses (Fandohan *et al.*, 2006). Animal feed stored under unfavorable conditions is prone to be infested by moulds either during pre-harvest in the fields or post-harvest in storage, such a fungal infection is accompanied, in the majority of cases, by contamination with mycotoxins (Fandohan *et al.*, 2006).

2.3.1 Factors affecting storage

Temperature

Temperature and moisture content of the animal feed are the two key features affecting the resulting quality of the grain, biochemical reactions, dry matter losses, allowable storage times and overall storage management of the grain (Jayas & White, 2003). The optimal temperature for growth of fungal species such as *Penicillium* and *Aspergillus* species is 25-30°C and 30-40°C, respectively. Hence, for safe storage of feed, both the moisture content and temperature of the grain and that of the surrounding be reduced and monitored (Jayas & White, 2003).

Moisture content

Moisture content plays an important role in the storage of feed; when grain has more moisture, it heats up and results in mould spoilage. Living organisms, such as moulds and insects, together with heat from the respiration of the grin itself will enhance water vapour, which in turn will lead to further deterioration of the grain. The higher the moisture content, the more susceptible the animal feed is to mould and insect deterioration during storage (Bankole & Adebanjo, 2003). Mycotoxins pose a risk to animal health and can affect livestock production for several species, but the risk to public health is considered low. In all cases, food of animal origin only marginally to the total human exposure to these toxins according to the Fertilizers, Farm feeds, Agricultural Remedies and Stock Remedies Act (Act 36 of 1947) and Regulations No. R. 70 of 12 February 2010.

Table 2.1: The maximum allowable levels of mycotoxins in animal feeds

Mycotoxins	Farm Feeds	Maximum content in mg/kg (ppm) relative to a farm feed with a moisture content of 120 g/kg	Maximum content in mg/kg (ppm) relative to a farm feed with a moisture content of 120 g/kg
Aflatoxin B_1	Feed ingredients except for:	0.05	50
	Ground nuts, copra, palm-kernel cottonseed, maize and products derived from the processing thereof	0.05	20
	Complete farm feeds for cattle, sheep, and goats except for:	0.05	50
	dairy cattle	0.005	5
	calves and lambs	0.01	10
	complete feeds for pigs and poultry (except young animals)	0.02	20
	other complete farm feeds (including pets)	0.01	10
	maize products intended for feedlot	300 000	300 000000
	Supplement/concentrates for cattle, sheep, and goats (except for dairy animals, calves and lambs	0.05	50
Fumonisin B_1	Horses	5	5000
	Pigs	10	10 000
	Beef and poultry	50	50 000
Ochratoxin A	Feeding stuffs on the full ration basis for Pigs	0.05	50
	Poultry	0.2	200
Zearalenone	**Feeding stuffs on full ration basis for:** Sows and piglets	5	5000
	Piglets	3	3000
	Calves and dairy cattle	0.5	5000

Relative Humidity

Relative humidity can be described as the amount of water vapour that is contained in the air as a proportion of the amount of water vapour required to saturate the air at the same temperature (Lawrence, 2005). If temperature increases, feed loses moisture to the surrounding air, thereby increasing the relative humidity. It has been observed that in most cereal grains, every 10° C rise in temperature causes an increase of about 3% in relative humidity (Shah *et al.*, 2010). Change in temperature and relative humidity not only promotes moulds growth but

also causes considerable nutrient losses of grain in the case of nutrients as reported by (Rehman & Mohandes, 2008).

Insects' infestation

Insect infestation is caused by improper post-harvest and the storage conditions are the foremost cause of deterioration and loss of agricultural product. The invasion of feedstuff decreases the quality, grade and market value of these agricultural products which makes it unsafe for animal and human consumption (Sivakumar *et al.*, 2014). Insects carry spores of mycotoxins-producing fungi from plant surfaces to the interior of the stalk or kernels which creates infectious wounds through their feeding habits (Munkvold, 2003b).

2.4 Animal feed storage types

Animal feeds are conducive to storage in upright bins, whereas other feedstuffs require storage areas such as commodity shed bay or being stored in containers, mud silos, or in bags (Feedlot, 2011). Generally, most of the storage facilities used by emerging farmers create inadequate storage conditions that consequently promote fungal infection and subsequent production of feed contamination especially mycotoxins (Hell *et al.*, 2000). The storage system to be used is determined by the bulkiness and associated storage space required for a given volume of feedstuff (Bryden, 2012). Storage life is an important consideration in feedstuff selection. For both commercial and emerging farms, use of jute, polypropylene, and polyethylene bags are commonly used to store animal feeds, with less than 1% of the traders storing their products in the recommended containers or bins (Chattha, 2015). Jute bags easily absorb moisture but allow good airflow while polypropylene and polyethylene are non-absorptive but trap heat within. Both farmers need to be aware of the physical characteristics of feedstuffs, such as high moisture content, that increases the likelihood of quality losses, deterioration, or spoilage. Feed storage facilities do not need to be fancy or expensive (Sudini *et al*., 2015). Improper drying, poor storage conditions, such as excessive heat and moisture, insects and other annoyances make feeds vulnerable to fungal infection and subsequent aflatoxin contamination during storage (Hell *et al*., 2000, Williams 2008).

2.4.1. Storage types:

Bunker or Trench Silos

These are generally the best option for storing large volumes of feedstuff such as corn silage which would be packed and covered in a bunker silo. Proper packing and covering are critical to reduce spoilage and ensuring a good-quality feed product for your livestock (Richards & Hicks, 2007). All bunker, trench or drive-over piles of feed should be covered with to create an anaerobic environment and minimize spoilage. Plastic should be inspected periodically, and any holes or tears should be repaired (Williams, 2008).

Plastic Silage Bags

Bags come in different sizes and lengths thus ensure space is large enough for equipment to move around to fill them. Inspection of bags and plastic bunker covers for tears or holes is important because oxygen penetration in these areas can cause additional spoilage (Gotlieb, 2002).

Oxygen-limiting Structure

Glass or steel silos are mostly commonly used by commercial farmers. They possibly create silos which aresed for storing feedstuff. Prior to storing the product in an oxygen-limiting storage structure, it is critical to contact the manufacturer to determine if the silo an handle the weight and density of the material (Galyean *et al*., 1992).

2.5 Type of storage facilities

Closed storage system

Closed storage should be kept clean, dry and at an appropriate temperature and humidity to minimize microbial growth because the value of the feed that is presented to animals depends on it (Hell *et al*., 1995). Feed spoils during storage, and this deteriorates quickly or slowly depending partly on its quality when received and stored on the farm. Therefore, building and storage containers should be well ventilated and monitored to minimize contamination or deterioration of feed and feed ingredients (Wagacha & Muthomi, 2008).

Open storage system

An open storage area is most likely to be contaminated or spoiled by mycotoxin or any microorganism that come in contact with storage of round bales outside. More damage occurs to less dense bales because of their tendency to squat (Munkvold, 2003a). Most round bales stored under trees are more likely to be damaged because they cannot dry well because the flat ground has reduced drainage and does not make a desirable storage site. Round bales stored

with the rounded sides touching have more damage because the shape stores moisture, causing spoilage (Williams, 2008).

Proper storage

To preserve quality feed in storage, it is necessary to prevent biological activity through adequate drying to less than 10% moisture. This can be done by elimination of insect activity which can increase the moisture content through condensation of respiratory gases, low temperatures, and inert atmospheres (Wagacha & Muthomi, 2008). In storage, the production of mycotoxins in grain and other feed ingredients can be avoided; this can be achieved by preventing growth of fungi and toxin production. In order to effect this ensure that moisture and temperature conditions do not favour the growth of toxin-producing moulds (Mycotoxins), avoid rodents and the infestation of insects. However, these contaminants occur naturally in the air in the field, making it very important to monitor for their occurrence in feedstuff and ingredients, given that mycotoxins are unequally distributed in commodities, it is essential to get an adequately representative sample and prepare the sample properly so reliable results are obtained.

2.6 Mycotoxins

Fungi are a continuous threat to livestock feeds of economic importance such as compound feeds, they may affect feed either directly by causing mechanical damage throughout the feeding, or indirectly by secreting and spreading mycotoxins such as aflatoxins in the case of aflatoxin-producing fungi (Sultana & Hanif, 2009). The common fungal genera contaminating compound feeds in South Africa are those belonging to the *Fusarium*, *Penicillium* and *Aspergillus flavus* and *Aspergillus parasiticus* elaborating the deterioration of compound feeds to reduce health effects and performance of those animals fed on such feeds (Iheanacho *et al.*, 2014). They are ubiquitous in nature and for some time, have become an increasing cause of life-threatening opportunistic disease. Various stresses like low-quality feed, naturally occurring toxic contamination in feedstuff, poor management, disease, climatic extremes and other constraints are ever present threats that can adversely affect performance and health of animals as well (Fink-Gremmels, 2008).

Formation of mycotoxins

Worldwide, approximately 25% of crops are affected by mycotoxins annually (Whitlow & Hagler, 2002), the data suggest routine exposure of animals to mycotoxins recently reviewed the challenges of mycotoxins contamination of small grains and corn grain are recommended greater preparedness to manage an expected increase in occurrence of mycotoxins associated with future climate and technological changes (Miller, 2008). 'Many species of these fungi produce mycotoxins in feedstuffs whereby moulds can grow and mycotoxins can be produced pre-harvest or during storage, transport, processing or feeding' (Mwanza, 2012).

Mould growth and mycotoxin production are related to plant stress caused by weather extremes, insects damage, inadequate storage practices and faulty feeding conditions as well as predisposing plants in the field, feed in transit or storage to mould growth and mycotoxin contamination (Coulombe, 1993). Moulds grow over a temperature range 10-40° C, pH range above 4-8 and moisture content greater than 13-15% which enhances most moulds to be aerobic and therefore high moisture concentration that exclude adequate oxygen can prevent moulds growth (Prandini *et al.*, 2009).

Pathological effects of mycotoxins

Pathological effects vary between different mycotoxins and different animals if the ingestion was of large amounts of toxin in a short period of time, this will cause acute toxicity leading to death while small doses in a prolonged length of time will results in chronic effects to the animal or side effect on humans (Marasas *et al.,* 1988).

In the feed manufacturing process the aflatoxins, trichothecenes, zearalenone, ochratoxins, and fumonisins are of particular interest, though the extent of harm each toxin can cause is highly species-dependent (Binder *et al.,* 2007). Mycotoxins when present in the diet, cause acute and/or chronic adverse health effects in animals and humans, depending upon the level consumed (Thieu *et al*., 2008).

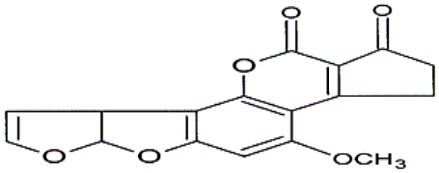
Table: 2.2– Overview of the major mycotoxins and their features in animal feeds (Bueno *et al.*, 2013)

Major classes of mycotoxins	Examples of mycotoxins producing fungi	Commodities	Effects observed in animal and human beings	Molecular formula	Chemical structure
Aflatoxin (B_1, B_2, G_1, G_2, M_1, M_2)	*Aspergillus flavus, Aspergillus parasiticus*	Fruits, rice, cheese, wheat, Corn, Oats, Sorghum, Hay, and forage	Carcinogenic liver damage and other adverse effects in humans, poultry, pigs, cattle and human toxicosis, internal	Aflatoxin: B_1 ($C_{17}H_{12}O_6$) B_2 ($C_{17}H_{14}O_6$) G_1 ($C_{17}H_{12}O_7$) G_2 ($C_{17}H_{14}O_7$) M_1 ($C_{17}H_{12}O_7$) M_2 ($C_{17}H_{14}O_7$)	**Figure 2.3:** Aflatoxins B_1 & B_2 **Figure 2.4:** Aflatoxin G_1 & G_2 **Figure 2.5:** Aflatoxin M_1 & M_2
Ochratoxin Ochratoxin A	*Aspergillus ochraceus, Penicillium verrucosum, Penicillium viridicatum*	Barley, Corn, Hay	Carcinogenic, kidney damage and other adverse effects in pigs and poultry. The immune system of mammalian species	Ochratoxin A $C_20H_{10}CINO_6$	**Figure 2.6:** Ochratoxin A
Fumonisins (B_1, B_2)	*Fusarium verticillioides (syn., moniliforme), Fusarium proliferatum*	Corn, Wheat, Maize, Barley, Oats, hay and forage	Suspected to cause human oesophageal cancer diseases of equines, pigs, and chicks	Fumonisins: B_1 ($C_{34}H_{59}NO_{15}$) B_2 ($C_{34}H_{59}NO_{14}$)	**Figure 2.7** Fumonisin (B_1, B_2)
Zearalenone	*Fusarium graminearum*	maize, barley, oats, wheat, ric e, and sorghum	lead to disrupted conception, abortion, cattle, pigs	Zearalenone $C_{18}H_{22}O_5$	**Figure 2.8** Zearalenone

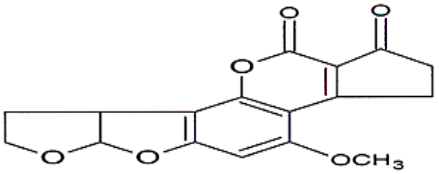
2.7 Mycotoxin description

Aflatoxin

Aflatoxins are secondary metabolites of *Aspergillus flavus* and *Aspergillus parasiticus* are frequently found in nuts, soya beans, maize and other plants, especially in areas with appropriate conditions of moisture and heat where these fungi are ubiquitous, they are basically produced at a temperature of 12-40° C and require 3-18% moisture (Duncan and Hagler, 2008). Sixteen aflatoxins have been identified, but only AFB1, AFB2, AFG1, AFG2, and AFM1 are routinely analyzed (Shephard, 2009). The letter B indicates that these aflatoxins have blue fluorescence to ultraviolet light (365 nm), while the letter G indicates the yellow-green fluorescence. Aflatoxins occur at temperatures that are between 25-35° C and the maximum yield in relation to the Aflatoxin B is attained between 28-30° C (Smalley, 1991).

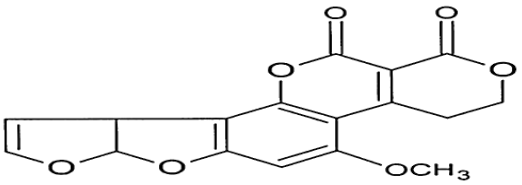


Aflatoxin B_1

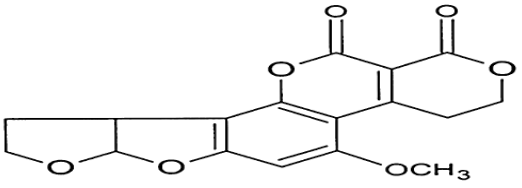


Aflatoxin B_2

Figure 2.3: Chemical structure of aflatoxin B (AFB1 and AFB), (McLean & Dutton, 1995)



Aflatoxin G_1



Aflatoxin G_2

Figure 2.4: Chemical structure of aflatoxin G (AFG1 and AFG2), (McLean & Dutton, 1995)

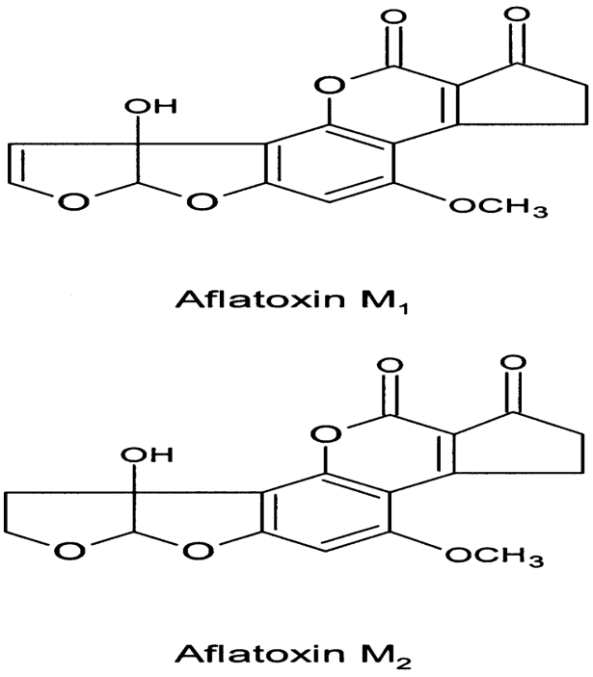


Figure 2.5: Chemical structure of aflatoxin M (AFM1 and AFM2) (McLean & Dutton, 1995)

Ochratoxin A

Ochratoxin A was discovered in 1965 as a metabolite of *Aspergillus ochraceus* during studies designed purposely at identifying new mycotoxin molecules (Van der Merwe *et al.*, 1965). Ochratoxin is a mycotoxin produced by certain fungi (*Aspergillus ochraceus* and *Penicillium verrucosum*), these moulds are the main contaminants in temperate regions where corn is the most contaminated product (Lancova *et al.*, 2008). These compounds are known for their nephrotoxic effects in poultry, they are considered to promote tumors in humans (Pavlović *et al.*, 1979). It has been found in the blood and other tissues of animals as we as including human milk (Afshar *et al.*, 2013).

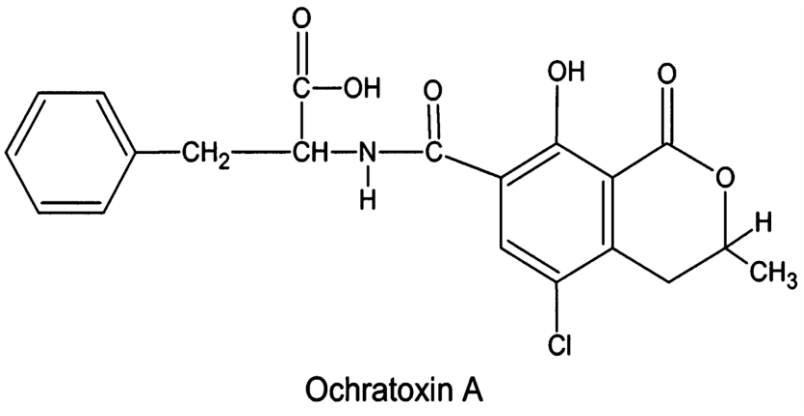


Figure 2.6: Chemical structure of Ochratoxin A (OTA), (Sweeney & Dobson, 1998)

Fumonisin

Fumonisins are a group of compounds that was first isolated in 1988 from *Fusarium* species. Commonly found in corn and in some other agricultural products, however, the Fusarium species are ubiquitous in moisture-damaged buildings, so contact of human population to fumonisins may also occur by air contamination (Murphy *et al.*, 1993). Fumonisins are reported to occur invisible on healthy grains. Currently, six fumonisins have been reported, FA1, FA2, and FB1, FB2, (Fig 2.7) FB3 and FB4. The A series are amides while the B series have a free amine (Gelderblom *et al.*, 1988) although fumonisin B1 (FB1) is the most abundant in naturally contaminated foods and feeds and generally comprises 75% (Jenkins *et al.*, 2000) of the total content .

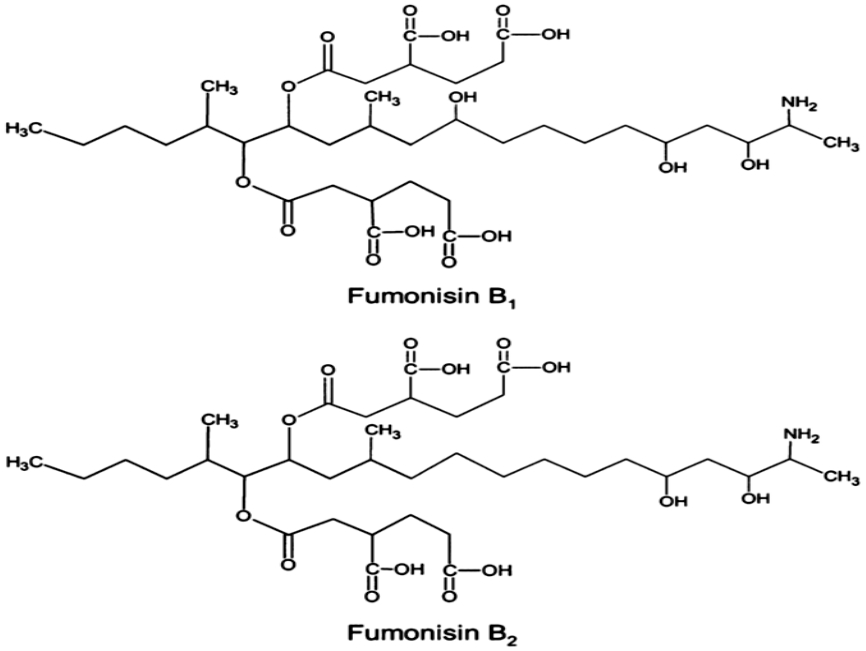


Figure: 2.7: Chemical structure of Fumonisin B1 and B2 (Sweeney & Dobson, 1998)

Zearalenone

Zearalenone is a secondary metabolite primarily produced by *F. graminearum* and *Fusarium* moulds using corn, wheat, barley, oats and sorghum as substrates. It is a non-steroidal compound that exhibits oestrogen-like activity in certain farm animals such as cattle, sheep, and pigs (Heidtmann-Bemvenuti *et al.*, 2011). Recent studies have demonstrated the potential for ZEN to stimulate the growth of human breast cancer cells containing oestrogen response receptors (Withanage *et al.*, 2001).

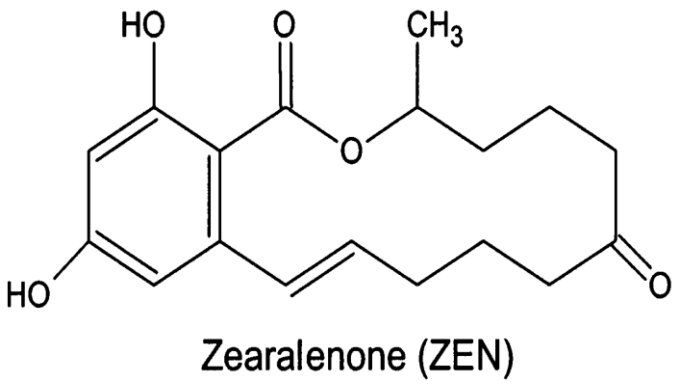


Figure 2.8: Chemical structure of Zearalenone (ZEN), (Sweeney & Dobson, 1998)

2.8 Major mycotoxins of concern for animals

Proper sampling procedures are pre-requisite for obtaining reliable results because of the heterogeneous distribution of mycotoxins in grains and other commodities (Whitaker *et al.*, 1979). Conventional analytical methods for mycotoxins include thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), Liquid Chromatography/Mass Spectrometry (LC/MS) and gas chromatography (GC). Most of these methods employ (ELISA), solid phase column (SPC) clean-up of extracts and immune-affinity (IAC) techniques to remove interferences to improve the measurement of mycotoxins.

2.8.1 Screening methods

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA methods for mycotoxins have been practiced for many years. The technology is based on the ability of a specific antibody to differentiate the three-dimensional structure of a specific mycotoxin. The direct competitive ELISA is commonly used in mycotoxin analysis (Chu, 1996). The principle of the ELISA test is the antigen-antibody reaction. The competitive assay format, in which the toxin competes with the enzyme conjugated to the toxin for specific immobilized antibodies, is often used in commercially available kits. Bound enzyme conjugate converts the substrate into a coloured, fluorescent or chemiluminescent active product. Another commonly used assay format is based on the competition between free and immobilized toxin for the binding sites for the toxin on the specific antibodies.

Immune-affinity column-based analysis (IAC)

The immune-affinity column (IAC) has been used widely for sample clean-up in mycotoxin analysis (Scott & Trucksess, 1997). The IAC contains an anti-mycotoxin antibody that is immobilized onto a solid support such as agarose gel in phosphate buffer, all of which is contained in a small plastic cartridge. The sample extract is applied to an IAC containing specific antibodies to a certain mycotoxin. The mycotoxin binds to the antibody and water is passed through the column to remove any impurities. Then by passing a solvent such as methanol through the column, the captured mycotoxin is removed from the antibody and thus eluted from the column. The mycotoxin in the methanol elute is then further developed by addition of a chemical substance to either enhance the fluorescence or render the mycotoxin fluorescent before measuring in a fluorometer (Trucksess *et al.*, 1991). Prior to adding a fluorescent enhancing chemical, the methanol solution can be used for HPLC analysis as well (Trucksess *et al.*, 1991).

Pre-treatment method

Solid Phase Extract (SPE) is by far the most popular technique currently used for analysis of fumonisin, aflatoxin B1, patulin, Ochratoxin in food and feed. The technology is based on chromatographic columns containing different bonding phases, ranging from C-18 (octadecylsilane), silica gel, anionic and cationic exchange materials to immunosorbents and molecular imprinted polymers (MIPs). The conventional SPE column retains the analytic on the adsorbent, the non-mycotoxin materials are eluted and then the mycotoxins are eluted. A sample extract is added to the sample reservoir and a rubber syringe plunger, or a similar device, is used to push the sample extract through the one-step SPE column. The purified extract collected at the lower end of the tube contains the mycotoxin, which can immediately be derivatized and placed in a fluorometer for analysis (Malone *et al.*, 1998).

2.8.2 Detection methods

Chromatographic techniques

Thin layer chromatography (TLC) is a method still broadly used for quantitative and semi-quantitative measurements of mycotoxins with detection by fluorodensitometry or visual procedures (0.01 ppm detection limit). TLC based on silica gel, F254 fluorescent silica gel or silica gel impregnated with organic acid has been reported to be applied for detection of common mycotoxins such as aflatoxins, citrinin, fumonisin (Lin *et al.*, 1998).

Gas chromatography (GC) is a technique applicable to the compounds that are volatile and thermo-stable. Detection is achieved by linking the system to mass-spectrometry (MS), flame ionization or Fourier transform infrared spectroscopy. Most mycotoxins are not volatile and therefore need to be derivatized by chemical reactions such as sialylation or polyfluoro acylation to be quantified. The method has been used to measure trichothecenes in fungal cultures in tandem with MS (Nielsen & Thrane, 2000). Due to its limitation to volatile and thermostable compounds, GC is not a technique suitable for commercial purposes.

High-performance liquid chromatography (HPLC) is widely accepted as an official method for the determination of toxins. It is applied in conjunction with UV, fluorescence, amperometric or spectrofluorometric detection. Both normal and reverse-phase HPLC is used for separation and purification (De Saeger *et al.*, 2003). A number of mycotoxins already have natural fluorescence (Ochratoxin, citrinin) and thus can be detected directly by HPLC-fluorescence (HPLC-FD), (Toscani *et al.*, 2007). Others, such as fumonisin, require derivatization that can be performed by employing o-phthaloyl aldehyde or 9-(fluorenylmethyl) chloroformate. Mycotoxin detection and analysis can be done using several analytical methods for the determination of major mycotoxins occurring in feedstuffs products. Advantages and disadvantages of traditional and emerging methods are reported in Table 2.3. Among the traditional methods, immune-affinity column clean-up coupled with HPLC is the most frequently used technique for the measurement of mycotoxins occurring in animal feed-based products. ELISA and other rapid antibody-based tests are generally used for screening purposes, although these methods often require confirmatory analyses (Krska & Molinelli, 2009).

Table 2.3 Advantage and disadvantage of traditional and emerging methods for mycotoxin analysis (Prieto-Simón *et al.*, 2007)

Method	Advantages	Disadvantages
Enzyme-Linked Immunosorbent Assay (ELISA)	Sample preparation, inexpensive equipment, high sensitivity, simultaneous analysis of multiple samples for screening, limited use of organic solvents.	Cross-reactivity with related mycotoxins matrix interference problems possible false positive/negative results, confirmatory LC analysis required.
Thin Layer Chromatography (TLC)	Can detect more than one mycotoxin	Slower than ELISA, proof of mycotoxin presence may require additional testing. May not work well for feeds other than grain.
Gas Chromatograph (GC)	Simultaneous analysis of mycotoxins, good sensitivity, may be automated (auto-sampler), provides confirmation (MS detector).	Expensive equipment, specialist expertise required, derivatization required, matrix interference problems, non-linear calibration curve, drifting response, carry-over effects from the previous sample, variation in reproducibility and repeatability
Liquid Chromatography/Mass Spectrometry (LC/MS)	Simultaneous analysis of mycotoxins, good sensitivity (LC/MS/MS), provide confirmation, no derivatization required.	Very expensive, specialist expertise required, sensitivity relies on ionization technique, a matrix-assisted calibration curve (For quantitative analysis),lack of internal standards
High-Performance Liquid Chromatography (HPLC)	Good sensitivity, good selectivity, good repeatability, may be automated (auto-sampler), short analysis times, official methods available.	Expensive equipment, specialist expertise required, may require derivatization

2.9 Prevention of moulds and mycotoxin in animal feeds

Mycotoxin contamination may occur in the field before harvest, during harvesting, or during storage and processing (Kabak, 2009). There are several ways by which farmers, suppliers, manufacturer and livestock producers can prevent the growth of mycotoxins contamination in animal feeds.

The practices that reduce mycotoxin contamination may differ depending on the climate of the region and the type of crop. Mycotoxigenic fungal growth can arise in storage as a result of moisture variability within the grain itself or as a result of moisture migration results from the cooling of grains located near the interface with the wall of the storage containers or silo (Kabak *et al.*, 2006).

Thus control of adequate aeration and periodical monitoring of the moisture content of silos plays an important role in the restriction of mycotoxin contamination during the storage (Heathcote & Hibbert, 1978). The moisture levels in stored crops are some of the most critical factors in the growth of mycotoxigenic moulds and in mycotoxin production. Prevention of mycotoxin formation in stored feeds is a major goal in controlling mycotoxicosis in livestock and poultry (Smith & Henderson, 1991).

In cases of light to moderate mycotoxin contamination, physical methods for cleaning the kernel surface, and hence removing the more heavily contaminated particulate matter, have proven effective in reducing mycotoxin concentrations. A range of chemical treatments have been applied to contaminated products and have been found to vary in their effectiveness at reducing mycotoxin concentrations in contaminated grain or feed (Galvano *et al.*, 2001).

The use of mould inhibitors is considered as one of the mould control method, whereby organic acids, especially propionic acid, form the basis of many commercial antifungal agents used in the stock feed industry and give excellent protection. Control of mould growth in feeds can be accomplished by keeping moisture low, feed fresh, equipment clean and use of mould binders. However, mould inhibitors are some of the numerous tools used in the complex process of controlling the growth of moulds and they should not be relied upon exclusively (Akande *et al.*, 2006).

CHAPTER III

METHODS AND MATERIALS

3.1 Sample collection

Feed samples were collected from Ngaka Modiri Molema District, clustered in four regions (East, North, West, and South) using the convenience sampling method where the sampled farms from each region were only the ones that were visited by the outreach program from the Department of Animal Health in North West University. The collection was done from May 2016 to January 2017, where a total of 100 animal feed samples (50-100g) were collected from emerging farms with 42 feed samples from a closed storage and 18 feed samples from open storage while 40 feed samples were from the corresponding commercial suppliers closed storage.

Figure 3.1: Animal feed storage facilities in Ngaka Modiri Molema District, South Africa, **(A)** Commercial feed suppliers – closed storage. **(B)** Used by Emerging farms – open storage and closed storage **(C)**.

3.1.1 Study area

Feed sampling was done during outreach organized by the Animal Health Department, in the Ngaka Modiri Molema District (NMMD) (Figure 3.2). During the pre-collection interview, questionnaires were distributed to farmers and suppliers to assess their knowledge and gather information regarding their feed storage facilities. Collected feed samples were then sent to the Animal Health Centre laboratory, North West University, for analysis.

(Sampling areas)

Figure 3.2: The map of Ngaka Modiri Molema District of North West Province.

3.2 Sample preparation and storage

Representative feed samples were finely grinded using a commercial grinder (IKA ® M20 Universal mill, USA), the fine sample was preserved in the Ziploc plastic bags and kept in the cold room (2°C) until the analysis was done. Samples were labeled and recorded.

3.3 Moisture content determination

Moisture content of the feed samples was determined by using the oven drying method. All feed samples were grinded, 1g of the feed sample was weighed in a crucible, then placed in a dry air oven, the samples were left in the oven to be dried at 105°C for 6 hours and the net weight of the dried samples determined (Undersander *et al*, 1993). Moisture content was calculated as shown below:

Moisture content:

(%weight basis) = (M0-M1)/M0×100

Where: M0 – initial weight, in grams of sample

Portion: M1 – final weight, in grams of dried sample content

Portion: M1 - final weight, in grams of dried sample content

3.4 Media preparation

Media (Potato Dextrose Agar, Malt extract and Sabouraud Dextrose agar) for culturing of fungi were purchased from MERCK, SA, and the individual media prepared according to manufacturer's instructions and autoclaved at 121ºC for 15 minutes. The prepared agar was allowed to cool to 45-50ºC after which 10mg/ml of chloramphenicol was added aseptically to inhibit the growth of bacteria. The molten agar (15ml) was then poured into sterile Petri dishes and allowed to solidify for inoculation of the serially diluted samples.

3.4.1 Serial dilution plate

Serial dilution technique was applied to determine the total fungal counts in animal feeds product samples, where 1 g of each composite sample was transferred into a screw-capped medical bottle containing 9 ml of phosphate buffered solution (PBS) in a dilution factor of 10^{-6} 1ml of each sample suspension was inoculated into Petri-dishes each containing 15ml of the prepared agar (PDA, MEA, and SDA). Plates were then incubated for the first 3-5 days at 28 ºC (Klich, 2002) for the fungal count and the results were expressed as counts of colony-forming unit (CFU/g). The plates were returned to the incubator till the 7^{th} day for growth of the different isolates. All plates were examined visually, directly and with a microscope (Aziz *et al*., 1998). The hyphae and conidia from each colony representing each fungal species were transferred aseptically into Malt Dextrose Agar (MEA) media for subculturing, then incubated at 28-30 ºC for 7days. Determination of each species of fungi was one using methods of Klich & Pitt (1988) for *Aspergillus* spp., Pitt & Hocking (1997) for *Penicillium* and *Fusarium* spp. Nelson *et al.,* (1993).

3.5 Molecular Identification

The genomic DNA of fungi was extracted using Fungal/Bacterial DNA extraction kit (Zymo Research Corporation, Southern California, and the USA) following manufacturer instructions, then DNA quantification, Polymerase Chain Reaction (PCR) was run, thereafter obtained DNA was sent for sequencing at INQABA Biotec in Pretoria, South Africa.

3.5.1 Fungal genomic Deoxyribose Nucleic Acid (DNA) extraction.

The genomic DNA was extracted from freshly prepared pure isolates from Malt Extract Agar (MEA). The pure isolates were scrapped from the agar surface and scooped into the 1.5ml ZR Bashing Bead™ tubes, freeze-dried and stored at -80 ºC. The DNA of the fungal culture were extracted by following the manufacturer's instruction as specified in the brochure by adding 100 µl of lysis solution in each sample and the lysis tube was placed in disruptor genie bead beater fitted with a 2 ml tube holder assembly (Scientific industries Inc., USA) and the process was done at maximum speed for 14 minutes repeated twice then after centrifuge lysed samples at 10 000 x g for 1 minute. The supernatant was transferred to a Zymo-Spin™ IV spin filter in a 1.5 ml Eppendorf tube and again centrifuged at 7000 x g for 1 minute. The contents were then filtered into a collection tube and 1200 µl of fungal/bacterial DNA binding buffer added and vortexed. The extracted mixture (800 µl) was transferred to a Zymo spin™ was transferred to a Zymo spin™ IIC column in the collection tube and again centrifuged at 10 000 x g for 1 minute and the supernatant was discarded. An aliquot (200 µl) of DNA pre-wash buffer was then added to Zymo spin™ IIC column in a new collection tube and centrifuged at 1000 x g for 1 minute. The filtrate was discarded while retaining the column, which was placed into a new tube. Some 500 µl of the DNA wash buffer was added to Zymo spin IIC column and again centrifuged at 10 000 x g for 1 minute. The Zymo spin™ IIC column was transferred into a sterile 1.5 ml Eppendorf tube and 100 µl DNA elution buffer was added directly onto the column matrix. This was then centrifuged at 10 000 x g for 30 seconds to elute the DNA. The eluted filtered DNA was stored at -80° C until further analysis.

3.5.2 DNA Quantity determination method

The quantity of the extracted genomic DNA was determined by measuring the absorbance (using 2µl of the samples) at 260 nm using NanoDrop 1000 spectrophotometer (Thermo Fisher Specific, Waltham, Massachusetts, US) following manufactures instruction. The machine was calibrated (blank) every after 10 samples by loading 2µl of TE buffer on the pedestal**.**

3.6 Gel electrophoresis

One-times (1 X) Tris/Acetate/EDTA (1x TAE) buffer was prepared by adding 4900ml of distilled water to 100 ml of 50 X TAE (375 ml of Tris-Cl, 28.55 ml of acetic acid, 50 ml of EDTA and 46.45 distilled water) and filled in the electrophoresis tank. Some 1.5 g of agarose (Fermentas Life Science, Lithuania) was prepared in 98 ml of 1x TAE buffer to give a 2% solution and melted in a microwave until boiled. The solution was allowed to cool to 60°C prior to the addition of 3µl ethidium bromide (Sigma-Aldrish, ST Louis, MO, USA) (10mg/1 in water to a final concentration of 05 mmol/ml) and thoroughly mixed. The gel was poured into the casting chamber (Bio-Rad Laboratories, California, and the USA) and the combs of desired sizes were inserted in such a way that no bubbles were caught under the teeth. After the gel was set, the combs were gently removed, and the gel was placed in the electrophoresis tank. Each PCR product 6 µl was mixed with 4µl of loading buffer and slowly loaded 10 µl into each of the wells in the gel with a sterile micropipette. A 6 µl of molecular marker 1 kilobase (kb) DNA ladder (Fermentas Life Science, Lithuania) was loaded in the first and last wells of each comb. The chamber was closed and ran at 450 V and 80 mA for 30 minutes. The gel electrophoresis was viewed on a UV transilluminator and photographed using the Chemidoc^{TM} MP imaging system (Bio-Rad Laboratories. California, USA).

3.7 Polymerase chain reaction (PCR)

The quality of extracted gDNA was accessed by subjecting them to 1.5% agarose gel electrophoresis and amplified using a PCR master mix and primer pair. The PCR primers including the forward primers ITS-1 (TCCGTAGGTGAACCTGCGG) and reverse primer ITS-4 (TCCTCCGCTTCTTGCTGC) were used to amplify the internal transcribed spacer (ITS) region of the extracted fungal DNA. Individual reactions had 1µl of DNA sample solution which was mixed with 13 µ1 master mix Taq DNA polymerase (Fermentas Life Science, Lithuania) and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR, 1µl of the primers i.e. ITS-1 (0.5 µl), ITS-4 (0.5 µl) each and 10 µl of nuclease-free water to make up total reaction volume of 25µl. The conditions for PCR were as follows: initial denaturation of DNA at 95°C for 3 minutes and then 35 cycles of denaturation at 94°C fir 1 minute, primer annealing at 58°C for 45 seconds and extension at 72°C for 1 minute and 30 seconds. A final extension of 10 minutes at 72°C was included and held at 4°C until samples were retrieved.

3.8 Detection and quantification of mycotoxins

A. Enzyme-linked immunosorbent assay (ELISA)

The Enzyme-linked immunosorbent assay (ELISA) was used as a screening method for Aflatoxin, Fumonisin, Ochratoxin, and Zearalenone in animal feed samples. An ELISA kit (RADISCREEN® R-Biopharm, AG Darmstadt, Germany) was used for the quantitative detection of the mycotoxins using the manufacturer's instructions.

Aflatoxin: Animal feed samples were grinded and 2 g of the sample were weighed into centrifugal screw cap vials, followed by the addition of 10 ml of 70% of methanol then vigorously shaken for 5 minutes using a horizontal shaker Edmund Buhler SM30 control (Hechingen, Germany). Then the extracted samples were filtered through Whatman No.1 filter paper for 5 minutes. Some 100µl of the extracted sample was diluted with 600 µl of. Only 50 µl of the diluted filtrated extract sample was poured respectively per well in the test. A volume of 50 µl of the standard solutions or prepared samples was added per well followed by the addition of 50 µl of the diluted enzyme conjugate to each well and gently mixed by shaking the plate manually and incubated at room temperature (20-25°C) for 30 minutes in the dark. Thereafter, the plates were washed three times with 250 µl of 1X wash solution then liquid was removed from the wells. Then 150 µl of 1X Antibody #2 solution was added to each well and the plate was incubated for 30 minutes at room temperature (20–25 °C). Then 100µl of chromogen/substrate was added to each well and mixed gently by shaking the plate manually and incubated at room temperature (20-25°C) for 15 minutes in the dark. Finally, 100 µl of stop solution was added to each well and mixed gently by shaking the plates manually. The absorbance was read at a 450 nm wavelength within 30 minutes of addition of stop solution and mixed gently by shaking the plate manually using an automatic Heales Model MB-580 microplate reader (Wellkang, London, UK).

Fumonisins: Animal feed samples were grinded and 2 g of the sample were weighed into a suitable 100 ml plastic container, then 12.5 ml of methanol/water (70/30; v/v) was added and shaken vigorously for 3 minutes using a Stuart® Orbital Shaker (Karlsruhe, Germany). The extract was filtered with Whatman No.1 filter paper and the filtrate diluted in the ratio of 1:14 with sample dilution buffer (Buffer 1) (contained in the kit). Sufficient numbers of microtitre wells were inserted into the microwell holder with the standard and sample positions recorded. Fifty microliters of the standard solutions or prepared samples were added into the wells followed by addition of 50 µl of the diluted enzyme conjugate and 50 µl of anti-FBs anti-body

solution to each well gently mixed shaking the plate manually and incubate for 30 minutes at room temperature (20-25°C) in the dark. Then poured out the liquid and tapped the microwell holder upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. The wells were washed by filling each of them with 250 µl of distilled water and liquid was again poured out. The washing process was repeated two times. Then 100 µl of chromogen and 50 µl of the substrate were added to each well and mixed gently by shaking the plates manually and incubated at room temperature (20-25 °C) for 15 minutes in the dark. Finally, 100 µl of stop solution was added to each well and mixed gently by shaking the plates manually. The absorbance was read at a 450 nm wavelength within 10 minutes of addition of stop solution using an automatic Heales Model MB-580 microplate reader (Wellkang, London, UK)

Ochratoxin A: Animal feed samples were grinded and 2 g of the sample were weighed into centrifugal screw cap vials, followed by the addition of 50 ml of 0.13 M sodium hydrogen carbonate buffer and vigorously shaken for 5 minutes using a horizontal shaker Edmund Bühler SM30 control (Hechingen, Germany). Then vials were centrifuged at 3500 x g for 15 minutes. About 50 µl of the standard solutions or prepared samples was added per well followed by the addition of 50 µl the diluted enzyme conjugate of each well and gently mixed by shaking the plates manually and incubated for 30 minutes at room temperature (20-25 °C) in the dark. Thereafter, the liquid was poured out of the wells and the wells were washed with 250 µl each and complete removal of liquid from the wells was ensured. Then 100 µl of chromogen/substrate was added to each well and mixed gently by shaking the plate manually and incubated at room temperature (20-25 °C) for 15 minutes in the dark. Finally, 100 µl of stop solution was added to each well and missed gently by shaking the plate manually. The absorbance was read at a 450 nm wavelength within 30 minutes of the addition of stop solution using an automatic Heales Model MB-580 microplate reader (Wellkang, London, UK).

Zearalenone: Animal feed samples were grinded and 2 g of the sample were weighed in suitable containers, followed by the addition of 25 ml of methanol/water (70/30; v/v) and shaken vigorously for 3 minutes using a Stuart® Orbital Shaker (Karlsruhe, Germany). The extract was filtered with Whatman No.1 filter paper and the filtrate diluted in the ratio of 1:7 with sample dilution buffer (Buffer 1 from the kit). Sufficient numbers of microtitre wells were inserted into the microwell holder with the standard and sample positions recorded. Some 50 µl of the standard solution or prepared samples were added to the wells followed by addition of 50 µl of the diluted enzyme conjugate. The contents were mixed gently by shaking the plate

manually and incubated for 2hours at room temperature (20-25°C) in the dark. Thereafter, the liquid was poured out of the wells and the microwell holder tapped upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. The wells were washed by filling each wells with 250 µl of distilled water and the liquid again poured out. The washing process was repeated two times. Then 50 µl of chromogen and 50 µl of the substrate were added to each well and mixed gently by shaking the plate manually and incubated at room temperature (20-25°C) for 30 minutes in the dark. Finally, 100 µl of stop solution was added at each well and mixed gently by shaking the plate manually. The absorbance was read at a wavelength of 450 nm within 30 minutes of addition of stop solution using an automatic Heales Model MB-580 microplate reader (Wellkang, London, UK).

B. Extraction and clean up using immune-affinity column for HPLC

The Immuno-affinity columns (IAC) were used as confirmatory tests where the following mycotoxins were extracted following the procedure of Biopharm, the manufacturer. The following products were used; EASI-EXTRACT® AFLATOXIN product code: RP71/RP70N and EASI-EXTRACT® ZEARALENONE product code: RP91/RP90

Aflatoxin

The extraction of aflatoxins was done according to manufacturer's instruction whereby 25 g of ground sample and 2.5 g sodium chloride was weighed into 600 ml beaker and 100 ml of 80 % methanol and blend at high speed using (IKA® M20 universal mill) for 5 minutes. The samples were then filtered through no.113 filter papers, 2 ml of the filtered sample was diluted with 14 ml of PBS solution into a clean vessel then the diluted filtrate was passed through the column at a flow of 2 ml per minutes to allow the antibody to capture the toxin in the column. The column was then washed with 20 ml of PBS at a flow rate of 5 ml per minutes and allowed the air to pass through to remove residual liquid in the column. The toxin was then eluted from the column at a flow rate of 1 drop per second using 1.5ml of 100 % methanol and followed by 1 ml of H_2O in a total volume of 2.5 ml into an amber glass. A 100µl of the toxin was then injected into the HPLC system.

Zearalenone

The extraction of Zearalenone was done according to manufacturer's instruction as follow; 12.5 g of ground sample was weighed into a 600 ml beaker and 125 ml of 75 % acetonitrile was added and blend at high speed for 2 minutes. The samples were then filtered through

no.113 filter papers, 10 ml of filtered samples was diluted with 40 ml of PBS solution and the pH was adjusted to 7.4 using sodium hydroxide. The diluted filtrate was passed through the column at a flow rate of 12.5 ml per minute to allow the antibody to capture the toxin in the column. Following that, the column was washed with 10 ml PBS at a flow rate of 5 ml per minute and allow the air to pass through the removal residual liquid in the column. Then the toxin was eluted from the column at a flow of 1 drop per second using 1 ml of 100% acetonitrile and collected in an amber glass vial and passed 1 ml of H_2O through the column and collect in the same vial in a total volume of 2 ml. Of this sample 100 µl was then injected into the HPLC system.

C. Pre-treatment method - Solid Phase Extraction (SPE)

Fumonisin

Solid phase extract is by far the most popular technique or method used to extract Fumonisin in the feed as described previously by Sydenham *et al*., 1992, Shephard *et al*., 2005 and Mwanza *et al*., 2014) where 10 g of animal feed samples were mixed with 40 ml acetonitrile/water (50/50; v/v). the extract was then filtered through Whatman No.4 and the extract was cleaned in solid phase extraction (SPE) isolate strong ion exchange (SAX) columns were used (International Sorbent Technology, UK). Column were pre-conditioned with 5 ml methanol and washed with methanol-water (70/30; v/v). The pH of the sample extract of 2 ml was adjusted to 5.9-6.5 with 1 M sodium hydroxide and further diluted with 4 ml methanol-water (70/30; v/v) and then loaded onto the SAX column. The cartridge were assembled on Water® vacuum manifold from Milford Massachusetts, the USA fitted to an air compressor. The flow rate was maintained at 1 ml per minutes with SPE manifold. The column was then washed with 5 ml methanol-water (70/30; v/v) and 5ml of methanol. The fumonisins were eluted with methanol-acetic acid and then eluent was evaporated to dryness at 50° C with a steam of nitrogen. The dry extract was stored until required and dissolved in methanol then ran HPLC.

D. High-Performance Liquid Chromatography for the detection and quantification of mycotoxins.

In this study, HPLC method was applied to determine and quantify FBs, AFs, and ZEA the exact concentration of the extracted mycotoxins according to the method used by Mwanza, 2014 with minor modifications. Briefly, each of the extracts from the IAC was dissolved in 200 µl of methanol, drying at each stage with a stream of warm air. The analyses of different

mycotoxins involved the use of specific mobile phases, emission and excitation wavelengths and different methods of detection. Table 3.1 summarises the comprehensive methods used to analyze the below-detected mycotoxins in feeds.

Aflatoxin detection and quantification

For Aflatoxin, the determination was done according to (Reiter *et al.*, 2009). Derivatisation used for AFs was KOBRA® CELL at 100 µl setting coupled with a coring cell. The mobile phase was composed of Methanol-Acetonitrile-Water (20:20:60, v/v/v) containing 119 mg of potassium bromide and 100 µl of nitric acid. Injection volume at 100 µl elution order was G_2, G_1, B_2, and B_1 pumped at the flow rate of 1.0 ml/minute. Fluorescence detector conditions were excitation 362 nm and emission 425 nm (B_1 and B_2), 455 nm (G_1 and G_2) with a flow rate of 1min/min.

Zearalenone detection and quantification

For Zearalenone, the analysis was done by fluorescent determination according to (Chilaka *et al.*, 2012) with modification. The extracts were serially diluted from initial concentration of 25 µg/ml to the lowest diluted concentrate of 0.00025 µg/ml. The mobile phase used was (Acetonitrile/Methanol/Water: 46/46/8, v/v/v). Some 100 µ1 was pumped at a rate of 2.5 ml/min injection volume per analysis. HPLC conditions were excitation at 355 nm and emission 440 nm. Column heater was constantly maintained at 40 ºC. The toxin concentration was determined by comparing the area of the standard peaks with the sample peak.

Fumonisins detection and quantification

For this study, Fumonisin was analyzed using (Egbuta *et al.*, 2015) with modification. The extracts were re-dissolved in 100 µ1 of methanol. The serially diluted concentration of FB_1 and FB_2 standards (100, 10, 0.1, 0.01 and 0.001 µg/ml) were pipetted into an HPLC vial and 400 µ1 of the derivatized agent O-pthaldialdehyde (OPA) was added and mixed. The column heater was at 40 ºC. The mobile phase was composed of methanol: saturated aqueous sodium dihydrogen phosphate (77/23, v/v), the injection volume was 100 µ1 pumped at a flow rate of 1.0 ml per minutes. The fluorescence detector was 335nm excitation and 440nm emission wavelength set. The pH of each extract was adjusted to 3.3 with o-phosphoric acid. FB_1 and FB_2 were identified by its constant retention time. The results were determined by comparing the peak area of the sample with the standard peak area.

Table 3.1 Mycotoxin detection method applied for Aflatoxin, Zearalenone and Fumonisin found in feeds on High-Performance Liquid Chromatography (HPLC**)**

Mycotoxin analyzed	Aflatoxin (Afs)	Zearalenone (Zea)	Fumonisins (Fum)
Extraction method	Immuno-Affinity Column (IAC)	Immuno-Affinity Column (IAC)	Solid Phase Extraction (SPE)
Sample amount extracted	25g/100ml	12.5g/125ml	10g/100ml
Standards concentration (µg/ml)	25µg/ml – 0.00025µg/ml	25µg/ml – 0.00025µg/ml	100 µg/ml – 0.001 µg/ml
Mobile phase	Methanol/Acetonitrile/H_20 20:20:60	Acetonitrile/H_20/Methanol 46:46:8	Methanol/Sodium di-hydrogen 77:23
Injection rate	100 µl	100 µl	100 µl
Flow rate	1.0 ml/min	1.0 ml/min	1.0 ml/min
HPLC detector	Fluorescent	Fluorescent	Fluorescent
HPLC conditions			
Excitation	362 nm	355 nm	335
Emissions	425 nm	440 nm	440

3.9 Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) plates were used for confirmation of mycotoxin found using the protocol of (Dutton & Kinsey, 1995). Aflatoxins were analyzed, using samples that were oven dried and dissolved in methanol then mixed vigorously using vortex machine. Aflatoxins standards were prepared $B_{1;}$ B2; G1; G2 and Mobile phase CEI = Chloroform: Ethyl Acetate: Propane-2-ol (90:5:5, v/v/v). Aluminum backed silica gel G plates (Merck Art 5553 20 × 20 cm) where lines were measured in 15 mm from each edge using a light sharp pencil. Some 20 µl spot portion of standards and samples were all implanted in the plates. About 10 ml of the solvent was poured in the tank and the plate was then placed in the origin at the bottom left-hand corner, allowing the solvent to reach the top of the plate and immediately removed it from the tank. The plate was dried using warm air the cooled. The plates were then inspected under short-wave ultraviolet 254 nm and long wave ultraviolet 365 nm (UV) light. We then drew around the fluoresced or absorbed spots and marked them then the distances were measured and the R_F value calculated.

3.10 Statistical analysis

Data obtained in this study were analyzed using IBM SPSS statistics 24 (version 24). The results for questionnaire data were analyzed using the descriptive method and the laboratory data analysis results were expressed as means and standard error of the mean (SEM). T-tests were used to determine the impact of storage type on the quality of feed with the significance level set at 50%. Analysis of Variance (ANOVA) was used when the groups s were more than two, for example, to determine the contamination per type of feed.

CHAPTER IV

RESULTS

Demographic information of emerging farmer

Gender of emerging farmers and commercial suppliers

This study presents the demographic results of the emerging farmers that were collected through an interview. All respondents were males 100% and no female emerging farmer was available nor interviewed. While about 33.3% of females are part of the feed suppliers around NMMD, with a variance of 66.7 % of male being owners of feed suppliers (annex 1)

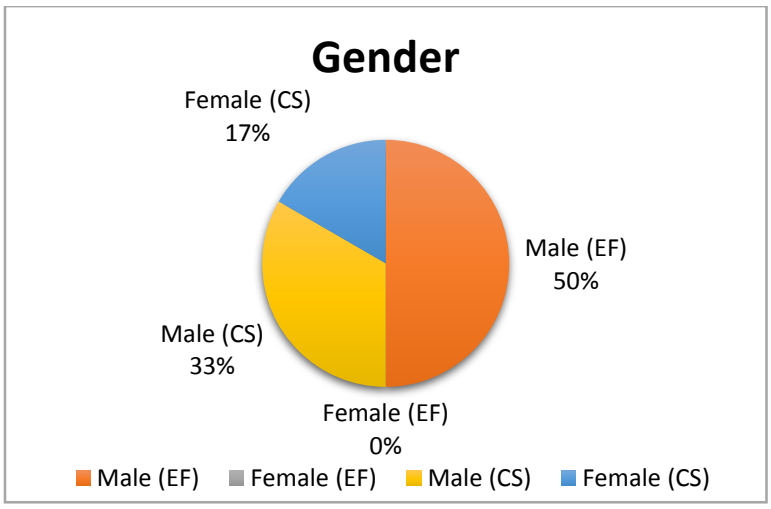


Figure 4.1: Gender of participated emerging farmers and commercial suppliers

The survey revealed that there are more farmers between age ranges 31-40 (50.0%) as compared to the other age groups (Fig 4.2). The age group 20-30 had 25.0%, while age group 41-50 made up 8.3% and the 51-60 group constituted 16.7%. This implies that the majority of the emerging farmers are young. In addition, data showed that among emerging farmers 41.7% only reached up to Secondary level, 50.0% at the Tertiary level and 8.3% had a Postgraduate level while among suppliers all of them reached the tertiary level. All emerging farmers were black and those who participated in this study 91.7% owned land and others were renting while all suppliers who participated in this study were renting their premises (Table 4.1).

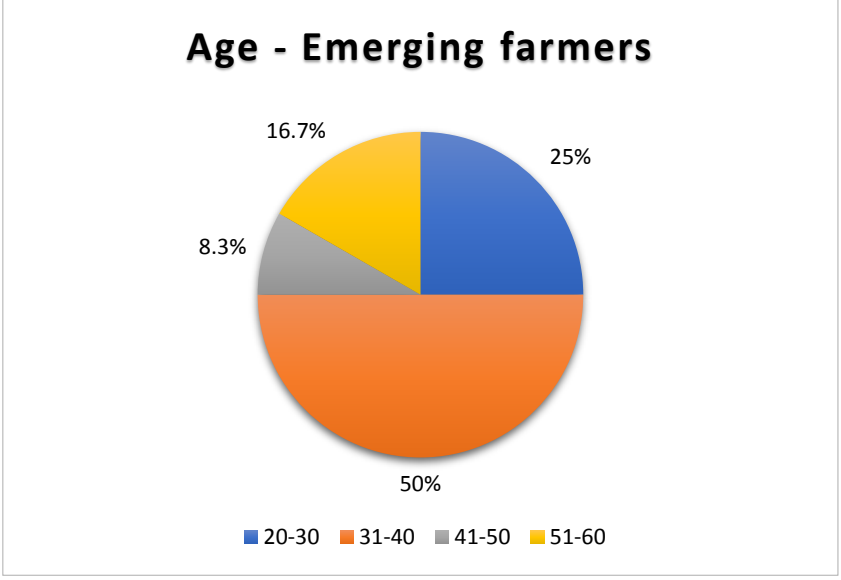


Figure 4.2: Age of participated emerging farmers

The below information indicate that majority of participated farmers, were black, self-employed and had educational level of 41.7% who went to secondary school. Moreover, feed commercial suppliers were white, married and had a tertiary level education compared to participated emerging farmers.

Table 4.1: Represent individual information from participated emerging farmers and commercial supplier

	Emerging farmers	Suppliers
Marital status	75.0% - Married	
	25.0% - Single	100.0% - Married
Educational level	41.7% - Secondary level	
	50.0% - Tertiary level	100.0% - Tertiary level
	8.3% - Post graduate level	
Race	100.0% - Black	100.0 – White
Working status	100% - Self-employed	100% - Self employed
Number of household	50.0% - 1-5 members	50.0% - 1-5 members
	50.0% - 5-10 members	50.0% - 5-10 members
Land ownership	91.7% - Owned	
	8.3% - Lease	100.0% - Lease

Livestock information of emerging farmers

All the farmers vaccinated their livestock through varying practices as shown in Figure 4.3, most (50%) used Animal Health Technicians to do the vaccination while 25% of the respondents animals are vaccinated by state veterinarians and the remaining 25% respondents vaccinate their animals by themselves

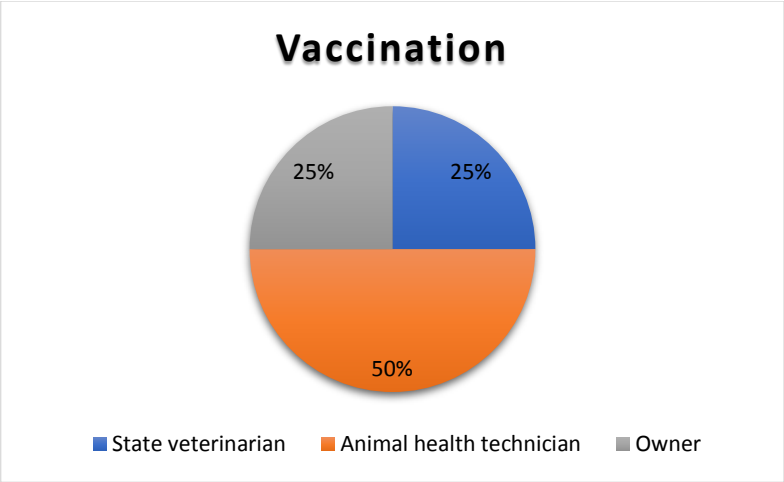


Figure 4.3: Summary of vaccination practice among emerging farmers

The figure 4.4 represents the accessibility to veterinary services by emerging farmers. Data showed that 16.7% of respondent received the visitation of the State Veterinarian on a monthly basis, about 41.7% are visited annually followed by 25% who sometimes gets visitations and 16.7% never gets visitations at all.

Figure 4.4: Percentage of veterinarian visits frequency received by emerging farmers.

The diagram below (figure 4.5) shows the different types of animal diseases encountered by emerging farmers were Tick-borne disease, lumpy skin disease and other diseases with 25% occurrence each.

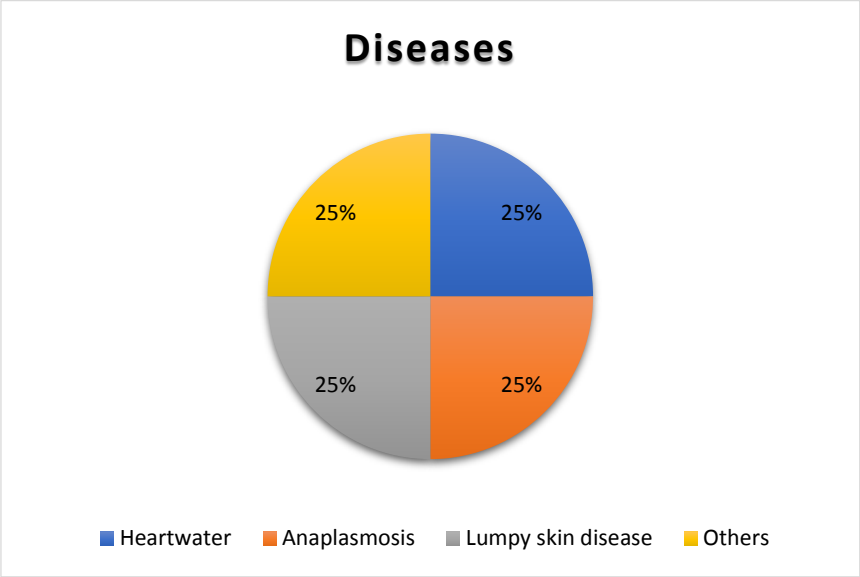


Figure 4.5: Diseases of domesticated animals encounter at emerging farm

Animal feeding information of emerging farmers

All the respondents of the study supplement their livestock by 100.0% because of the weather conditions experienced in the Ngaka Modiri Molema district. They purchase their feed, none of them prepare their own feeds. The study shows that respondents prefer mixed feed at a rate of 100% than any other type of feed being fed to livestock animals.

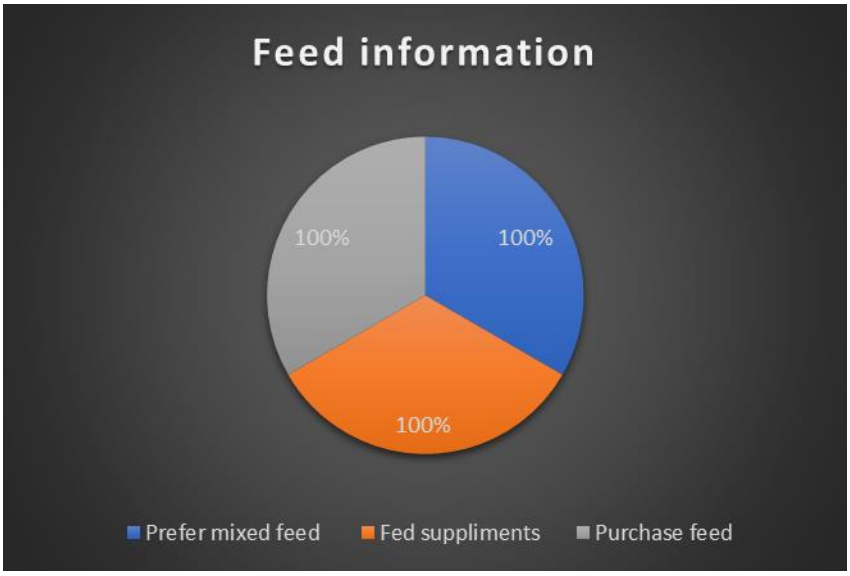


Figure 4.6: Feed information of what participated emerging farmers prefer to use for feeding their animals

The North-West Province is regarded as one of the few provinces that does not experience rain frequently, this study shows that the respondents experience dry weather condition, more than any other weather conditions. Farmers kept their feeds at room temperature between (22- 28 °C) (Fig. 4.7).

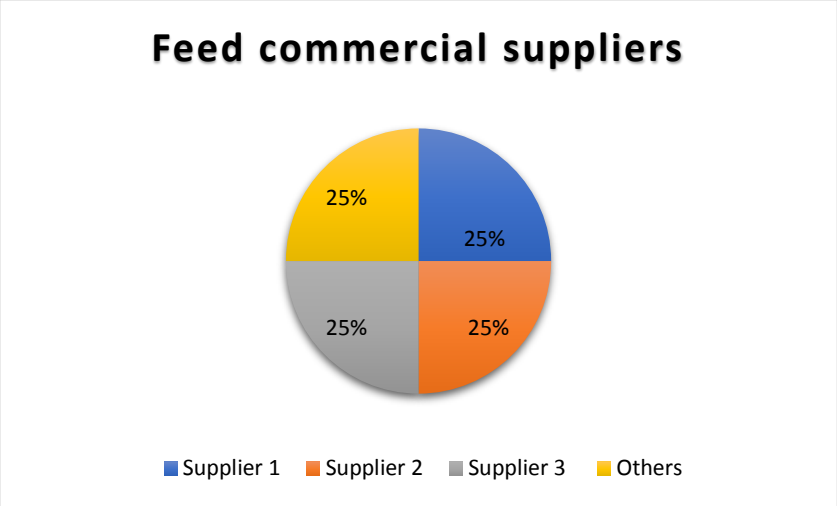


Figure 4.7: Feed commercial suppliers of where emerging farmers buy their feed used for feeding their animals

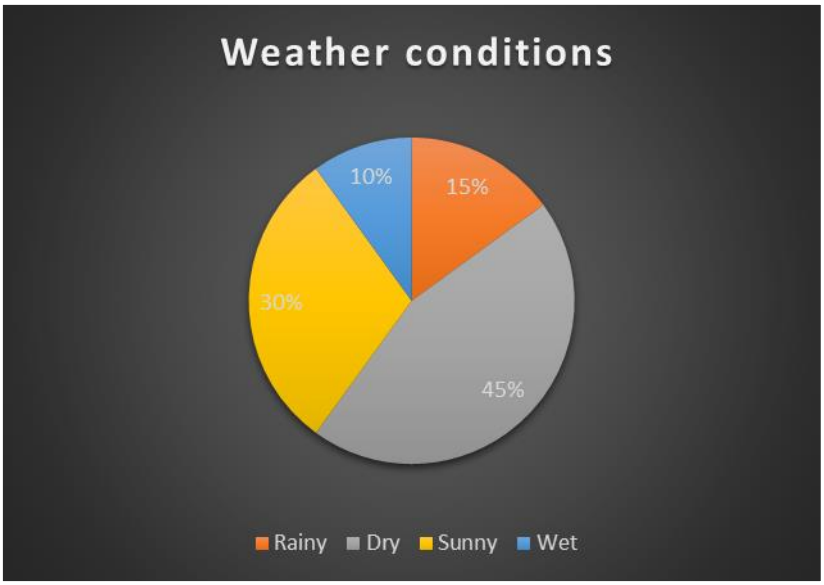


Figure 4.8: Weather condition and temperature in of which participated farmers regulated their feed storage.

Two major types of storage facilities were commonly used by the farmers; whereby 41.7% respondents used open storage facilities and 58.3% used closed storage facilities to preserve their feedstuff (Fig. 4.9).

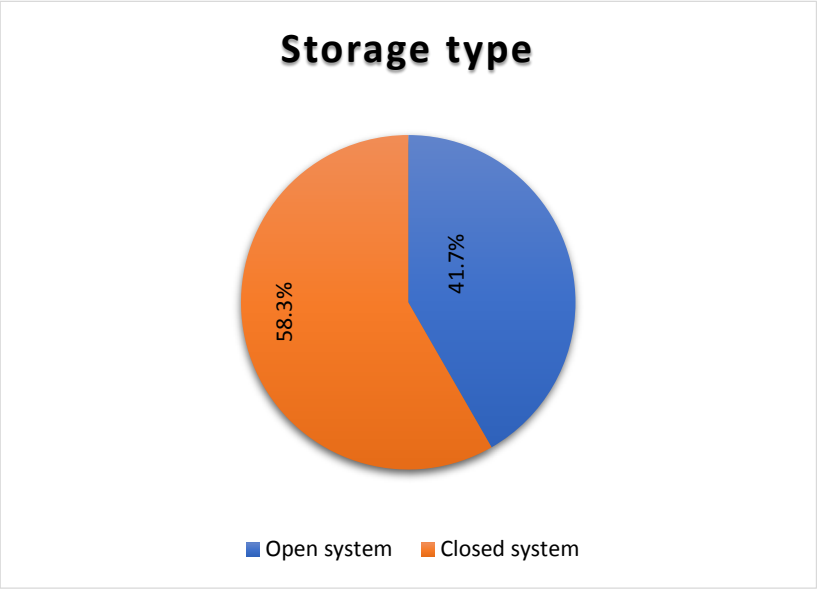


Figure 4.9: Summary of the type of storage facilities used by participated emerging farmers to store their animal feed

In this study, 58.3% respondents confirmed that they preferred using bags as a package method to preserve their feed, and about 41.7% used dustbins as another method to preserve their animal feed (Fig. 4.10).

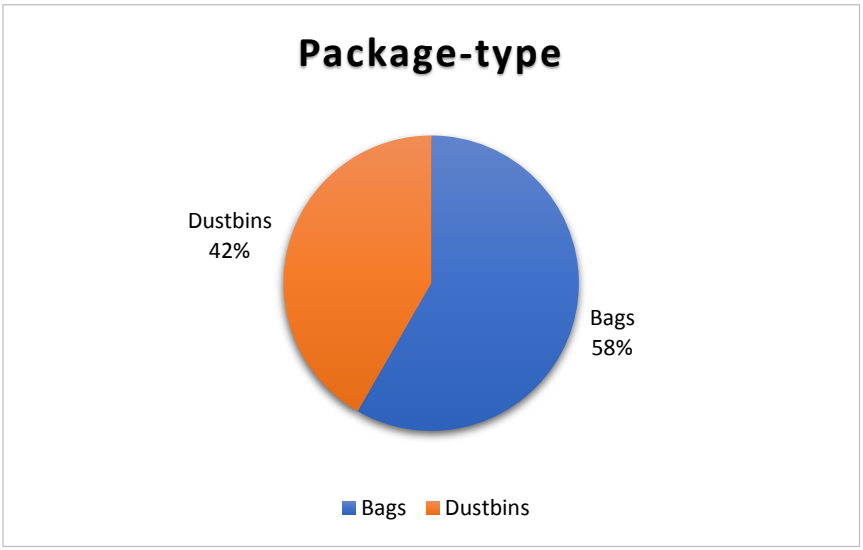


Figure 4.10: Types of storage packaging method used by participated emerging farmers for preservation of animal feeds.

Figure 4.11 represents the duration of storage periods for the feeds. Some 50.0% of the respondents keep their feed for about 1-3 weeks while the rest 50.0% keep their feed for 1-3 months.

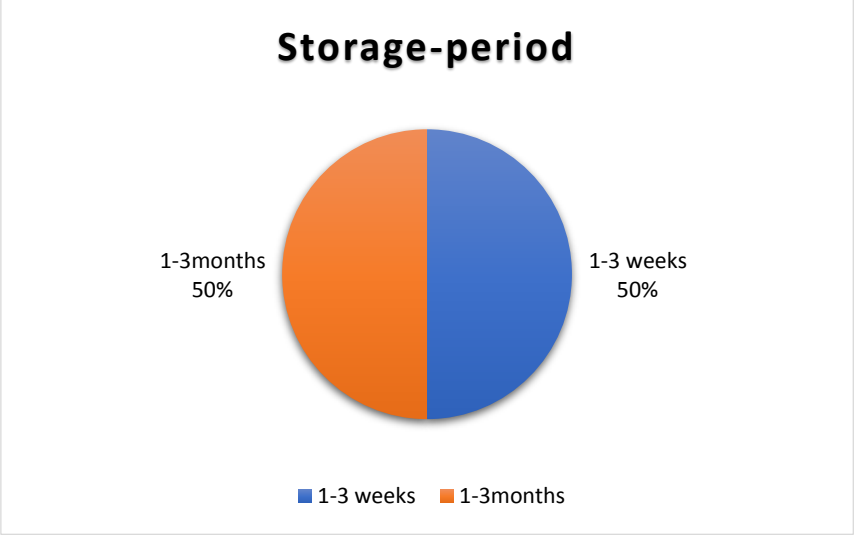


Figure 4.11: Duration of Storage time used by participated emerging Farmers

In this study, 58.3% of respondents confirmed that they knew and understood the issue of feed contamination, while 41.7% did not know or understand anything related to it. However, 100.0% of the respondents did not have an idea about what "mycotoxins" and their side effects were (Fig. 4.12). Hence 100.0% of the respondents were showing some interest in knowing more about mycotoxins and their impacts on animal and human health.

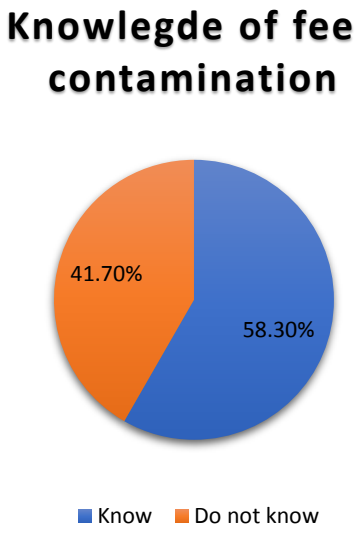


Figure 4.12: Emerging farmers who had Knowledge about feed contamination with regards to storages

Commercial feed suppliers information

Data from the survey among feed suppliers showed that most of the supplying companies have been operating for about 50 years and more (66.7%), and 33.3% have been operating for 1-10 years.

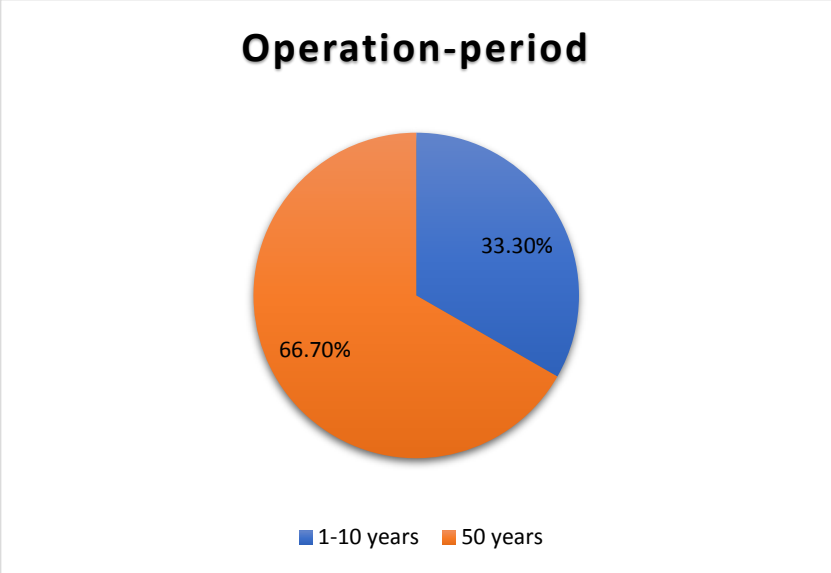


Figure 4.13: Duration of operation of participated feed commercial suppliers

Individual companies supply different products from different brands to different suppliers and they also supply for private companies, farmers, and the public in general (Fig. 4.14).

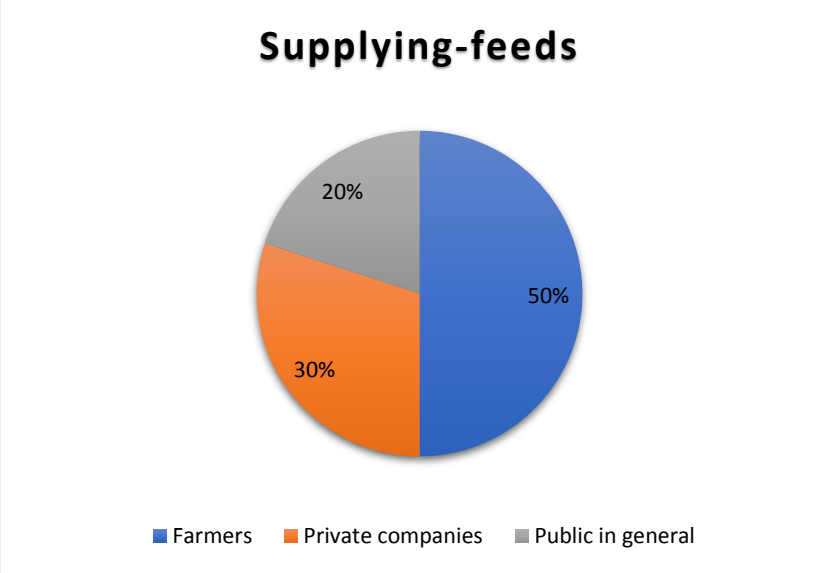


Figure: 4.14: Participated feed commercial suppliers who supplied animal feeds for emerging farmers

Most companies are registered under the adapted standard law of Act 36 of 1974. Their products are inspected annually, such that the floor that they keep their feedstuff on is also inspected (Fig. 4.15).

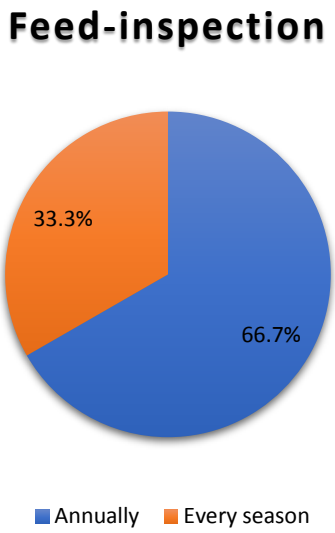


Figure 4.15: Represent inspection routine done on feed commercial supplier products.

Different companies use different hygienic method to keep their floors sanitized. About 66.7% still use general cleaning (Green cleaning), and 33.3% uses machines to clean their storage facilities.

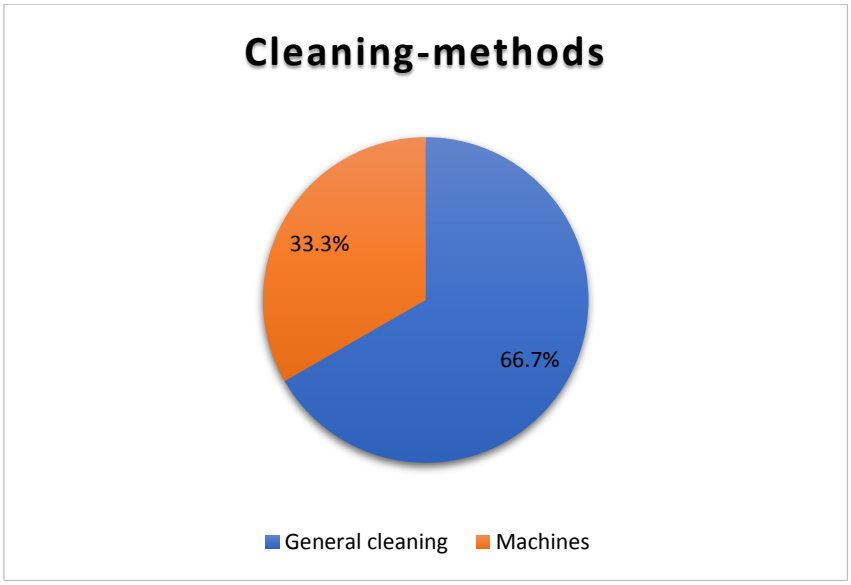


Figure 4.16: Represent cleaning method used by participated feed commercial suppliers

The storage facilities are closed system where dry and animal feed products are kept at a room temperature (22-28ºC) but this temperature is not usually monitored. The products are kept for as long as possible before its expiring date.

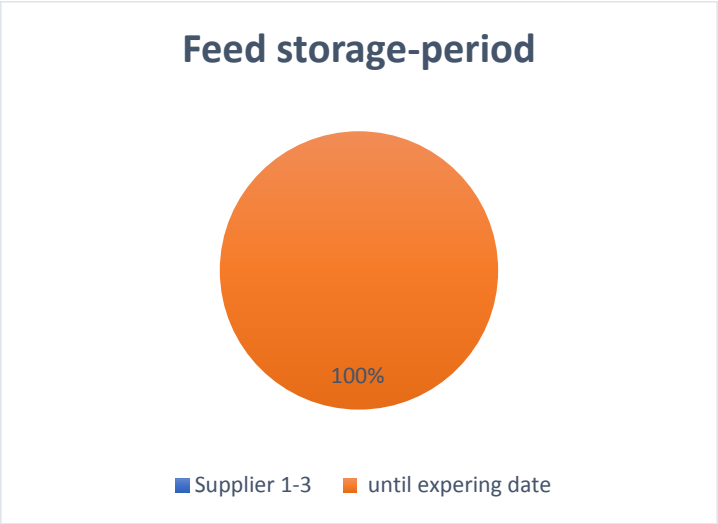


Figure 4.17: Represent feed storage period used by participated feed commercial suppliers

In this study windows and storage doors are used as ventilation systems, hence the door (50%) and windows (50%) are always open to maintain ventilation in the storage facility, but rodents enter the storage area, even though the suppliers try to keep them away from the storage area by using poison that has a repellent. All (100%) of the suppliers use bags to keep their product, others use the method of shelving on the wood to avoid rodents and insects invading into the bag pack.

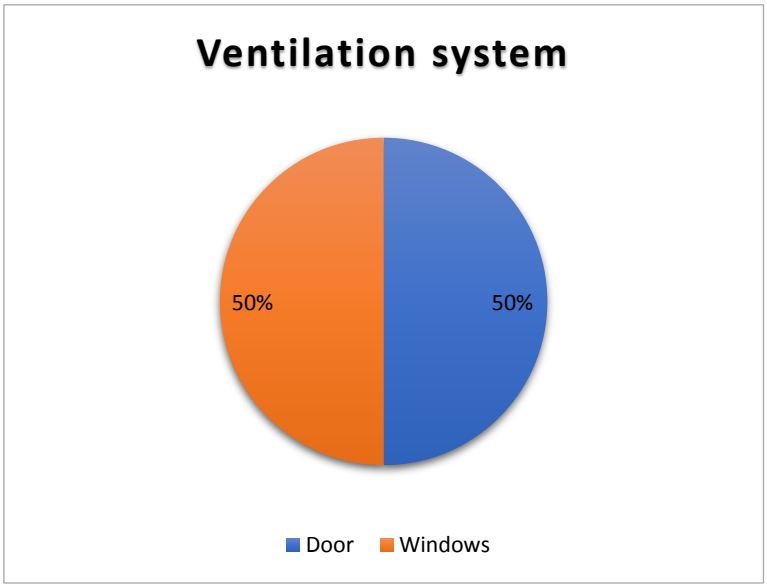


Figure 4.18: Represent ventilation system used by participated commercial feed suppliers

4.2 LABORATORY TEST RESULTS

Moisture content

For this study moisture content of feed samples were determined individually, and the results are demonstrated in Table 4.2 below.

Table 4.2. Summary of moisture content results from all type of collected storage facilities and different animal feed samples

Emerging farmers - Closed storage		Emerging farmers- Open storage		Commercial feed supplier – Closed storage	
Animal feed sample:	**Moisture content (%)**	**Animal feed sample:**	**Moisture content (%)**	**Animal feed sample:**	**Moisture content (%)**
Mixed feed	5.2	Molasses	6.5	Sheep and goats' pellets	6.2
Broiler - grower	4.2	Mixed feed	5.7	Kalori 3000	3.6
Broiler - starter	4.5	Winter lick	10.4	Swine lactation	6.9
Sunflower mix	3.4	wheat bran	5	Lucern	4.1
Pig pellets	5.8	Ruminant lick	7.3	Broiler – finisher	8.5
Sheep and goats pellet	6.9	Sheep and goats' pellets	4.5	Bone meal	3.5
Winter lick	9.8	Peanut Hay	6.7	Beef finisher	4.8
Dairy cattle pellets	4.3	Layer - starter	5	Winter lick	5.1
Calve - grower	4.5	Lebrun hay	6.3	Feed lime	1.2
Sheep and goats' pellets	8.8	Sheep and goats' pellets	7.1	Summer lick	7.7
Layer – mash	4.2	Pig pellets	6.9	Broiler – grower	6.1
Mixed feed	9.8	Molasses	5.1	Pig – grower	5.4
Dairy cattle pellets	4	Ruminant lick	6.1	Mealies	6.8
Sheep and goats' pellets	6.1	Mixed feed	8	Salt	7.5
Beef lick	4.6	Winter lick	9.1	Molasses	5.2
Phase D	3.3	Salt	1.6	Pig – grower	13.4
Molasses	5.5	Grass Hay	3.6	Boiler – starter	4.7
Mixed feed	9.9	Silage	16.3	Bovine phase D	6.9
Ruminant lick	5.1			Winter lick	7.2
Cattle pellets	3.6			Winter breaker	6
Peanut Hay	1.6			Molasses	6.6
Mixed feed	8.5			Ruminant lick	7.1
Winter lick	10.3			Pig – finisher	5
Winter lick	7.6			Corn	6.8
Blue buffalo grass	6.2			Beef – grower	6.6
Mixed feed	8.6			Layers – starter	1.3
Mixed feed	8.7			Broiler – starter	7.4
Swine - starter	6.9			Cattle pellets	6
Swine - starter mix	5.8			Layers – grower	5.9
Swine lactation	7.9			Cattle – pellets	6.1
Swine - weaner	8.7			Sheep and goats pellets	5.2
Soar - boar	7.6			Salt	3
Pig - grower	8.2			Mealies	4.8
Corn	10.3			Beef – finisher	9.1
Pig - lactation	7.5			Ruminant lick	4.6
Pig - grower	8.3			Drought pellets	8.1
Sheep and goats pellets	8.6			Molasses meal	6.1
Cattle pellets	7.5				5.2
Winter lick	7.3				8.5
Layer - mash	6.5				9.8
Peanut Hay	6.3				
Salt	0.3				

Fungal isolation

The findings of fungal cultures and colony count from animal feed samples were expressed as colony forming units (CFU/g). The results are summarized in Tables 4.3 and 4.4, which show that for the farmers, there is only a slight difference in the mean Cfu/g across the two storage types with the closed storage having more Cfu/g. Also, the mean Cfu/g from the suppliers' closed storage is slightly more than the one from the open as well as the closed storages from the farmers.

Table 4.3: Represent the relationship between the type of storage and the mean fungal contamination (Cfu/g).

	Type of storage	N	Mean	Std. Deviation	Std. Error Mean
Colony counter fungal unit (Cfu/g) (x10^6	Closed storage (Farmer)	18	2.17	0.985	0.232
	Open storage (Farmer)	14	2.57	1.158	0.309
Colony counter fungal unit (Cfu/g) (x10^6	Closed storage (Supplier)	14	2.64	0.842	0.225

Table 4.4: T-test results for Storage Equity Means of fungal colony unit (Cfu/g).

	t-test for Equality of Means		
	T	**Df**	**Sig. (2-tailed)**
Colony counter fungal unit (Cfu/g) (x10^6 (Farm Open vs Farm Closed)	-1.068	30	0.294
Colony counter fungal unit (Cfu/g) (x10^6 (Farm Open vs Supplier Closed)	-.187	23.743	0.853
Colony counter fungal unit (Cfu/g) (x10^6 (Farm Closed vs Supplier Closed)	-1.473	29.687	0.151

T-test, DF – Degree of freedom, Sig – p-value*

Results obtained in this study revealed that there were no significant differences P>0.05 between the mean of storage systems using t-test (Table 4.4). The supplier's closed storage was more contaminated compared to closed and open storage of farmers whilst between the farmers' storages the closed storage was slightly more contaminated than that open one.

Animal feed that was sampled showed fungal contamination isolated and identified from this study revealed about 78% tested positive from both storages (open and closed) of emerging

farms and (closed storage) commercial suppliers. The fungal strains isolated from sampled feeds are summarised in Table 4.5 – 4.7. The results revealed that major mycotoxins found were from *Aspergillus spp* 42%, (*A. Niger, A. flavus, A oryzae, A. fumigatus, A. terreus, A. clavatus,* and *A. parasiticus*). Several *Aspergillus spp* were from a closed storage (12%) and least from open storage (8%) facilities of emerging farms and more from commercial supplier's storage facilities (22%). Around 26% of the *Penicillium spp, (P. verrocusom, P. polonicum,* and *P. chrysogenum*) were obtained from closed storage and only 20% from open storage facilities of emerging farms whilst 6% were collected from commercial supplier's animal feed storages. *Fusarium spp* amounted to 10%, *(F. oxysporum)* was only found in open storage facilities of one of the sampled emerging farms and other similar fungal species included *Talaromices flavus, Byssochlamys spectabilis, and Cladosporium asperulatum* 4%, 2% from open storage collected in emerging farms storage and 2% from closed storage facility of commercial suppliers. Below images (Figure 4.19 - 4.20) showing the mouldy contamination found in collected animal feed samples.

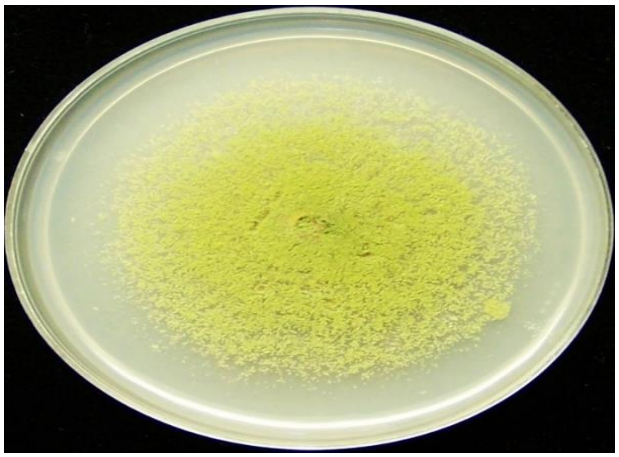


Figure 4.19: A plate culture of *Aspergillus flavus* growing on Potato Dextrose Agar at 25°C for 7 days

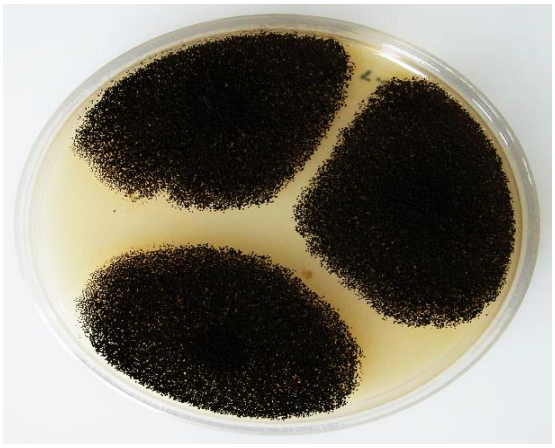


Figure 4.20: A plate culture of *Aspergillus niger* growing on Potato Dextrose Agar at 25°C for 7 days

Table 4.5: A summary of fungal strains of contaminated feed samples from emerging farms

Closed storage – Emerging farms (EF)			
Farm identification	**Contaminated sample**	**Isolate strains**	**Cfu/g**
Farm 2		*Aspergillus Flavus Penicillium*	$2x10^6$
Farm 7		*polonicum*	$3x10^6$
Farm 9	Winter lick (5)	*Penicillium brevicompactum*	$2x10^6$
Farm 10		*Cladosporium asperulatum*	$1x10^6$
Farm 12		*Talaromytes spectabilis*	$3x10^6$
Farm 7	Corn (2)	*Aspergillus oryzae*	$3x10^6$
Farm 11		*Aspergillus parasiticus*	$4x10^6$
Farm 2	Pig pellets (9)	*Aspergillus terreus*	$1x10^6$
Farm 11		*Aspergillus amstelodami*	$3x10^6$
		Aspergillus clavatus	$3x10^6$
		Penicillium rubens	$1x10^6$
Farm 2	Starter-broiler (2)	*Penicillium polonicum*	$2x10^6$
Farm 3		*Aspergillus flavus*	$3x10^6$
Farm 7	Molasses (2)	*Byssochlamys spectabilis*	$1x10^6$
Farm 10		*Aspergillus flavus*	$3x10^6$
Farm 2	Sheep and goats'	*Byssochlamys spectabilis*	$1x10^6$
Farm 3	pellets (4)	*Aspergillus flavus*	$2x10^6$
Farm 6			
Farm 12			
Farm 7	Peanut hay (1)	*Sarocladium zeae*	$1x10^6$

Table 4.6: A summary of fungal contamination of feed contamination of feed samples of open storage from emerging farms.

Open storage – Emerging farms (EF)			
Farm identification	**Contaminated sample**	**Isolate strains**	**Cfu/g**
Farm 1	Winter lick (2)	*Cladosporium asperulatum*	$1x10^6$
Farm 8		*Penicilium brevicopactum*	$4x10^6$
Farm 11	Silage (1)	*Aspergillus flavus*	$5x10^6$
		Aspergillus nomius	$2x10^6$
		Aspergillus fumigatus	$3x10^6$
		Penicilium verrucosum	$3x10^6$
Farm 5	Pig pellets (2)	*Aspergillus niger*	$3x10^6$
		Aspergillus parasiticus	$2x10^6$
Farm 1	Mixed molasses	*Aspergillus amstelodami*	$2x10^6$
Farm 5	(2)	*Byssochlamys spectabilis*	$1x10^6$
Farm 1	Sheep and goats'	*Aspergillus fumigatus*	$2x10^6$
Farm 5	pellets (3)		
Farm 4			
Farm 4	Lebrun hay (1)	*Fusarium oxysporum*	$4x10^6$
Farm 1	Salt (3)	*Cryptococcus paraflavus*	$2x10^6$
Farm 8		*Penicillium chrysogenum*	$2x10^6$
Farm 11			

Table 4.7: A summary of fungal contamination of feed contamination of feed samples of Commercial suppliers.

Closed storage – Commercial feed suppliers (CFS)			
Feed supplier identification	**Contaminated sample**	**Isolate strains**	**Cfu/g**
Supplier 1	Broiler - finisher (2)	*Aspergillus tubingesis*	$2x10^6$
	Sheep and goats' pellets (3)	*Aspergillus oryzae*	$2x10^6$
	Swine lactation (2)	*Penicillium rubens*	$4x10^6$
	Summer lick (2)	*Aspergillus fumigatus*	$2x10^6$
Supplier 2	Cattle pellets (2)	*Aspergillus clavatus*	$3x10^6$
	Pig grower (2)	*Aspergillus terreus*	$4x10^6$
	Starter broiler (2)	*Aspergillus amstelodami*	$3x10^6$
	Mealies (1)	*Aspergillus niger*	$3x10^6$
	Winter lick (2)	*Aspergillus nomius*	$3x10^6$
	Corn (2)		
Supplier 3	Molasses meal (1)	*Aspergillus fumigateaffinis*	$2x10^6$
	Beef leak (2)	*Ascomycota species*	$1x10^6$
	Salt (1)	*Aspergillus fumigatus*	$2x10^6$
	Beef finisher (2)	*Talaromyces flavus*	$3x10^6$
	Calve – grower (2)	*Penicillium chrysogenum*	$3x10^6$
	Winter lick (2)		

Fungal identification

Samples were selected based on their morphological similarities from each sample per farm and type of storage facility. For identification of cultured materials in this study, agarose gel electrophoresis of animal feed samples was used (Fig 4.21), Polymerase Chain Reaction (PCR) selected positive samples stained with ethidium bromide and photographed under UV light.

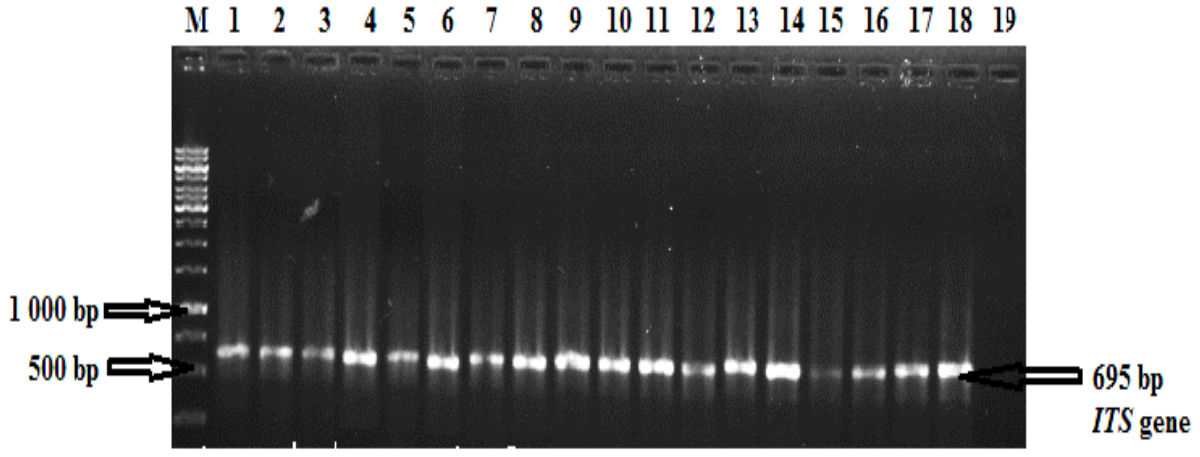


Figure 4.21: Electrophoresis on a 1.5% agarose gel of PCR amplified ITS gene. Lane M: Molecular weight marker (1kb); Lane 1-18 (*ITS* gene fragments from DNA extracted from feed samples), Line 19: Negative control

Table 4.8: Fungal genera contaminants of animal feeds collected from emerging farm storage and commercial supplier storage facilities around Ngaka Modiri Molema District

Storage type	Sample = n	Fungal genera					
		Aspergillus	***Fusarium***	***Penicillium***	***Talaromytes***	***Cladosporium***	***Byssochlamys***
Open storage (EF)	**18**	11 (61.1%)	1 (5.5%)	8 (44.4%)	-	-	-
Closed storage (EF)	**42**	30 (71.4%)	-	19 (45.2%)	6 (14.3%)	3 (7.1%)	2 (5%)
Closed storage (CFS)	**40**	25 (62.5%)	2 (5%)	11 (27.5%)	4 (10%)	3 (7.5%)	1 (2.5%)
Total percentage (%)	**100**	**66**	**3**	**38**	**10**	**6**	**3**

Table 4.9: Summary of the fungal strains isolated from collected animal feed storage facilities were confirmed by Polymerase Chain Reaction

Isolated strains	No. of samples contaminated	Incident rate %
	***Aspergillus* species.**	
A. flavus	14	21
A. oryzae	7	10
A. terreus	4	6
A. fumigatiaffinis	2	3
A. fumigatus	4	10
A. clavatus	4	6
A. parasiticus	11	16
A. tubingensis	3	4
A. niger	9	13
A. nomius	3	4
A. amstelodami	2	3
	***Fusarium* species.**	
F. oxysporum	5	10
	***Penicillium* species.**	
P. verrucosum	13	34
P. chrysogenum	9	23
P. polonicum	7	18
P. rubens	3	8
P. brevicompactum	6	16
	***Talaromyces* species.**	
T. spectabilis	3	3
T. flavus	4	4
	***Cladosporium* species.**	
C. asperulatum	1	1
	Other fungal strains	
Ascomycota species	2	2
Paecilomyes species	2	2
Sarocladium zeae	2	2
Acremonium strictum	2	2

4.10: Represent the similarity identification of fungal strains with the accession number with reference from NCBI database

Sample identity	Fungal identity	Percentage similarity (%)	Accession Number
Seq1	*Aspergillus flavus*	100%	MG647833
Seq2	*Cryptococcus paraflavus*	99%	MG647834
Seq3	*Penicillium polonicum*	99%	MG647835
Seq4	*Byssochlamys spectabilis*	99%	MG647836
Seq5	*Aspergillus flavus*	99%	MG647837
Seq6	*Penicillium brevicompactum*	99%	MG647838
Seq7	*Fungal sp*	95%	MG647839
Seq8	*Aspergillus fumigatiaffinis*	98%	MG647840
Seq9	*Aspergillus oryzae*	99%	MG647841
Seq10	*Cladosporium asperulatum*	99%	MG647842
Seq11	*Penicillium verrucosum*	99%	MG647843
Seq12	*Aspergillus terreus*	99%	MG647844
Seq13	*Aspergillus parasiticus*	99%	MG647845
Seq14	*Aspergillus clavatus*	99%	MG647846
Seq15	*Penicillium chrysogenum*	99%	MG647847
Seq16	*Trichoderma Orientale*	99%	MG647848
Seq17	*Aspergillus amstelodami*	99%	MG647849
Seq18	*Aspergillus clavatus*	99%	MG647850
Seq19	*Aspergillus terreus*	100%	MG647851
Seq20	*Aspergillus sp*	99%	MG647852
Seq21	*Aspergillus nomius*	99%	MG647853
Seq22	*Aspergillus oryzae*	99%	MG647854
Seq23	*Aspergillus fumigatus*	95%	MG647855
Seq24	*Aspergillus Niger*	99%	MG647856
Seq25	*Aspergillus flavus*	99%	MG647857
Seq26	*Fusarium oxysporum*	99%	MG647858
Seq27	*Fungal sp*	99%	MG647859
Seq28	*Aspergillus sp*	99%	MG647860
Seq29	*Penicillium rubens*	99%	MG647861
Seq30	*Ascomycota sp*	96%	MG647862
Seq31	*Aspergillus tubingensis*	99%	MG647863
Seq32	*Paecilomyces sp*	98%	MG647864
Seq33	*Byssochlamys spectabilis*	99%	MG647865
Seq34	*Aspergillus sp*	99%	MG647866
Seq35	*Aspergillus Niger*	99%	MG647867
Seq36	*Aspergillus oryzae*	99%	MG647868
Seq37	*Aspergillus fumigatus*	99%	MG647869
Seq38	*Sarocladium zeae*	99%	MG647870
Seq39	*Acremonium strictum*	99%	MG647871
Seq40	*Talaromyces spectabilis*	98%	MG647872
Seq41	*Talaromyces flavus*	96%	MG647873
Seq42	*Penicillium sp*	99%	MG647874
Seq43	*Aspergillus flavus*	99%	MG647875

Spp* = Species

4.3: Phylogenetic trees

Phylogenetic tree 1: Aspergillus species

The evolutionary history of the Aspergillus isolates was inferred using the Neighbour-joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 38.08814141 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the member of base substitutions per site. The analysis involved 47 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 541 positions in the final dataset. Evolutionary analyse were conducted in MEGA7 (Kumar *et al*., 2016).

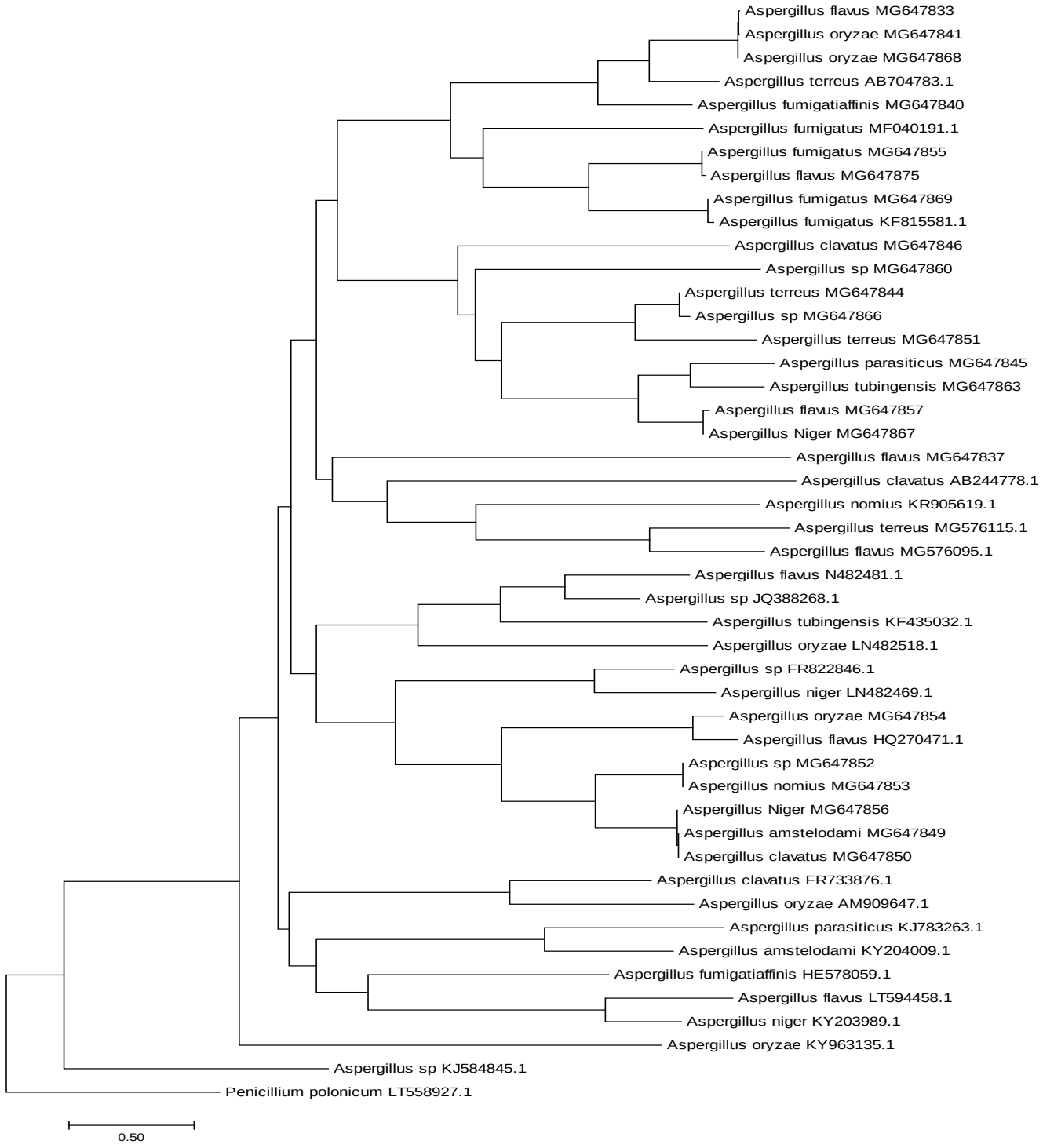


Figure 4.22: The phylogenetic tree of ***Aspergillus*** **species** isolated in animal feed and confirmed by PCR

Phylogenetic tree 2: Penicillium species

The evolutionary history of the *Penicillium* species was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 17.57300025 is shown. The tree is drawn to scale, with branch length in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The analysis involved 13 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 546 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016)

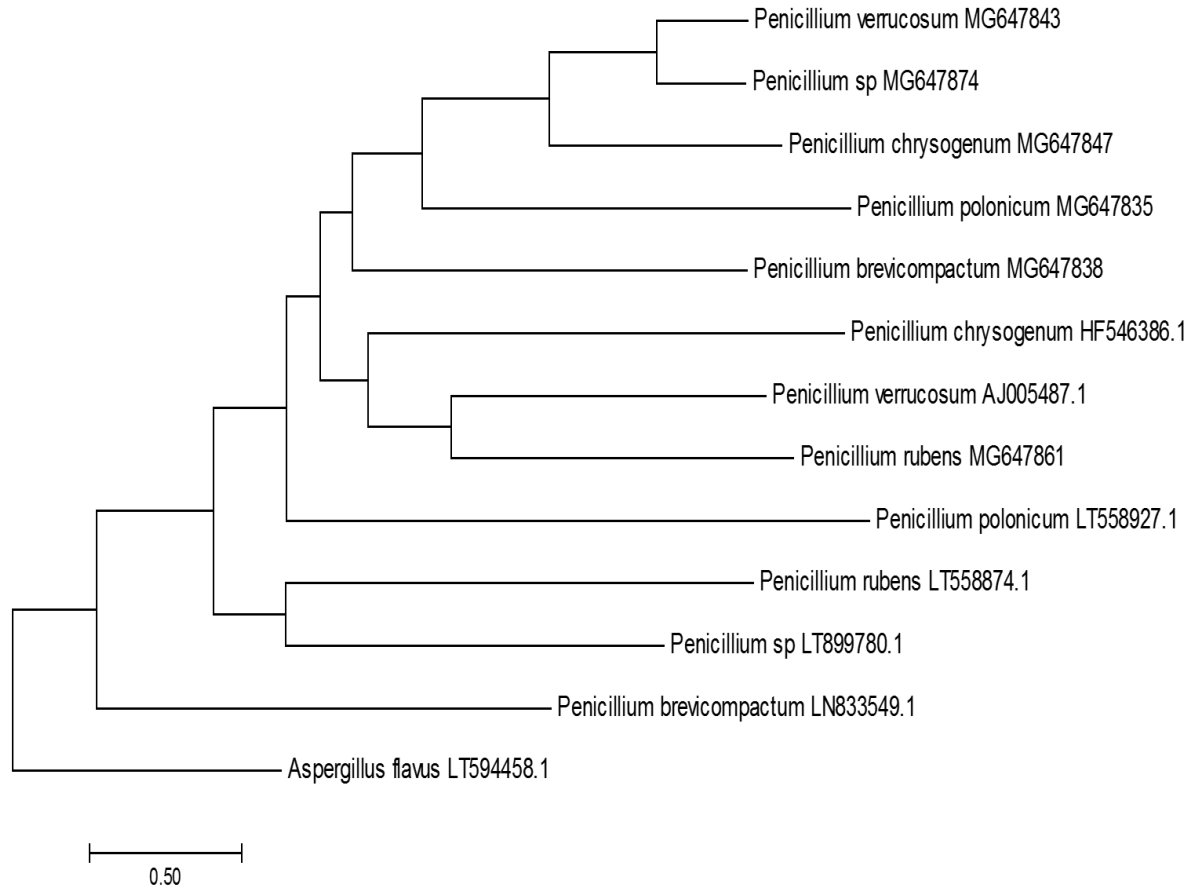


Figure 4.23: A phylogenetic tree of ***Penicillium*** **species** isolated in animal feed and confirmed by PCR

Phylogenetic tree 3: Talaromyces species

The evolutionary history of the *Talaromyces* was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 15.20493635 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The analysis involved 5 nucleotide sequences. Codon positions include were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 519 positions on the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

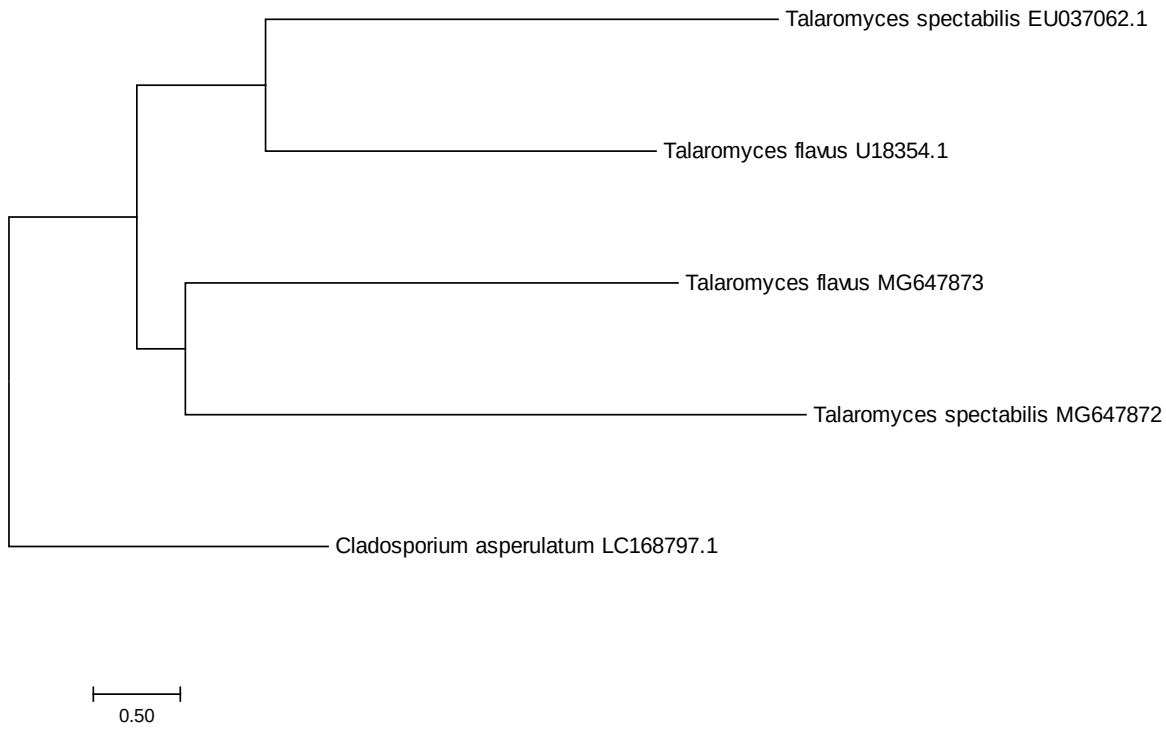


Figure 4.24. A phylogenetic tree of ***Talaromyces*** **species** isolated in animal feed and confirmed by PCR

Phylogenetic tree 4: Fungal species

The evolutionary history of fungal species was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 16.0367198 is shown. The tree is drawn to scale, with branch length in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The analysis involved 5 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 21 positions in the final dataset. Evolutionary analyses were conducted. There was a total 421 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

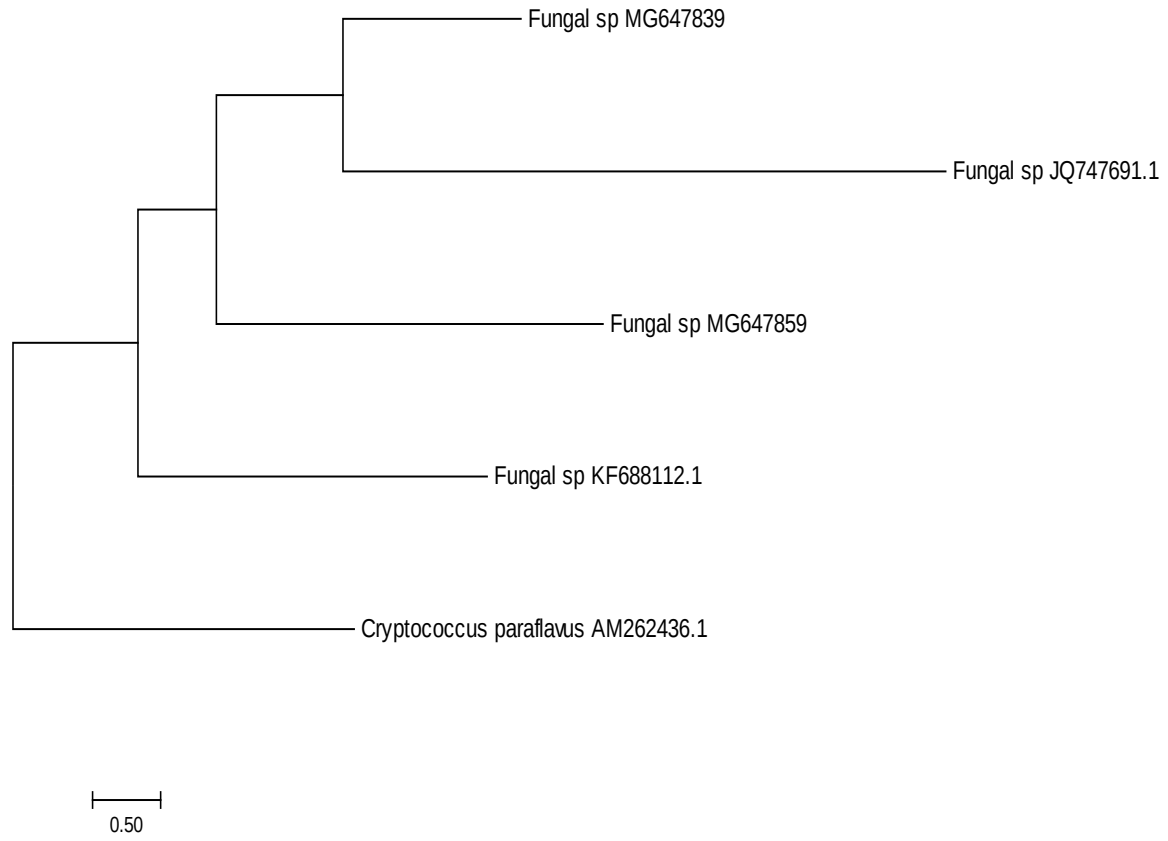


Figure 4.25: A phylogenetic tree of ***fungal*** **species** isolated in animal feed and confirmed by PCR.

Phylogenetic tree 4: Byssochlamys species

The evolutionary history of the *Byssochlamys* was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 4.00645571 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*. 2004) and are in the units of the number of base substitutions per site. The analysis involved 5 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 558 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

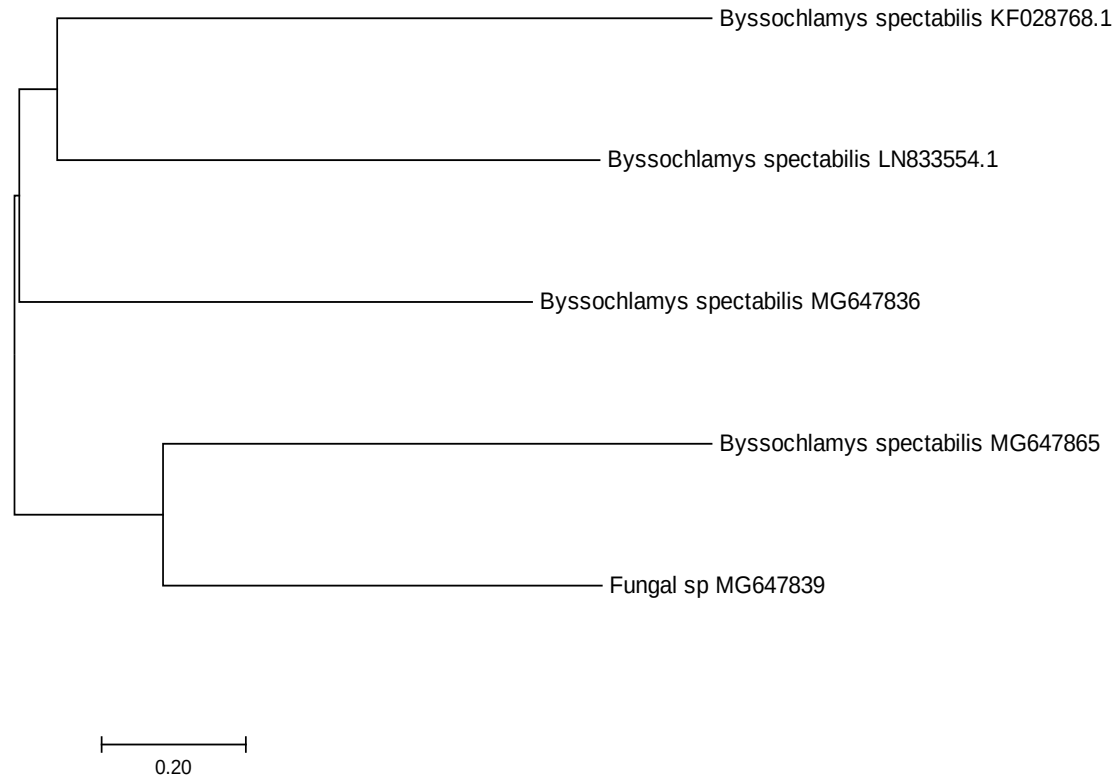


Figure 4.26: A phylogenetic tree of ***Byssochlamys*** **species** isolated in animal feed and confirmed by PCR.

4.4 Mycotoxins detection and quantification

Enzyme-linked immunosorbent assay (ELISA).

In this study a screening test for aflatoxins was carried out using ELISA and the standard calibration curve is shown in Figure 4.27. The detection of four mycotoxins (Aflatoxin, Fumonisin, Zearalenone and Ochratoxin A) in feed samples were analysed using ELISA and results were compared to the Maximum Tolerated Limits (MTLs) in ppb according to (Mazumder & Sasmal, 2001) in animal feeds.

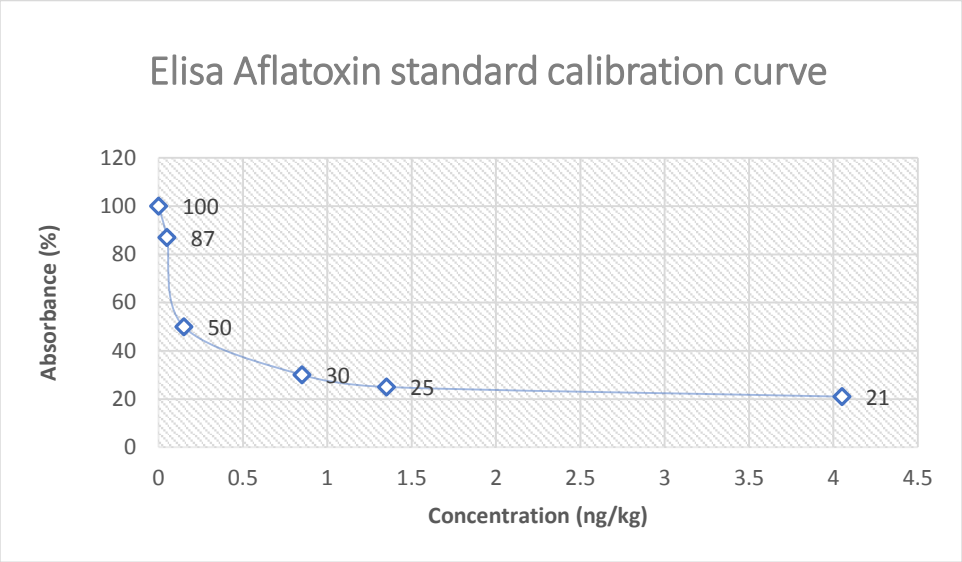


Figure 4.27: Represent the aflatoxin standard calibration curve obtained from the $R^2 = 0.9986$ and regression value of y = -2.1976x + 99.916

The results showing AFBs contamination in animal feed samples from three collected storage facilities are shown in Table 4.11. The highest AFBs were observed from emerging farm's closed storage with a mean of 632.03 ppb and the lowest mean been 575.96 ppb from an open storage facility of emerging farms.

Table 4.11: Summary of aflatoxins contamination in animal feed analysed in open and closed storage facilities of both emerging farmers and commercial suppliers as determined by Enzyme-linked immunosorbent assay (ELISA)

Type of storage	Mycotoxins	Mean (ppb)	Range (ppb)	Std. Dev	>MRL
Open storage – EF	AFB_S	575.96	0.51 - 0.71	176.03	7(36.8)
Closed storage – EF	AFB_S	632.03	0.72 – 0.55	49.77	22(53.6)
Closed storage – CFS	AFB_S	618.91	0.75 - 0.56	51.95	27(67.5)

Ef – emerging farms, cfs – commercial feed suppliers*

The results showing (Total aflatoxin and MRL_sa) contamination in animal feed samples from three collected storage facilities are shown in Table 4.12. The highest TOTAL_af was observed in closed storage of feed commercial suppliers. Mean, Standard Deviation and Standard Error Mean were determined regarding the results of total number of positive feed samples.

Table 4.12: Comparison of the relationship between the type of storage and the mean mycotoxin contamination (Total aflatoxin and MRL_sa)

	Type of storage	N	mean	Std. Deviation	Std. Error Mean
TOTAL_AF	Open storage (Farmers)	20	471.45	1180.867	264.05
	Closed storage (Farmers)	39	540.462	1603.389	256.748
MRL_SA	Open storage (Farmers)	20	0.47	1.175	0.263
	Closed storage (Farmers)	39	0.541	1.606	0.257
TOTAL_AF	Open Storage (Farmers)	20	471.45	1180.867	264.05
	Closed Storage (Suppliers)	36	16465.64	32545.53	5424.255
MRL_SA	Open Storage (Farmers)	20	0.47	1.175	0.263
	Closed Storage (Suppliers)	36	16.464	32.55	5.425

Data obtained and analysis of TOTAL_af on Table 4.13 – 4.14 showed that the differences noted in the preceding table are significant (p-value > 0.05) on the mean contamination of the samples collected from the farmers.

Table 4.13: Revealed that t-test statistics (P< 0.05) it is significant in the mean contamination between the storage system used by the suppliers (closed storage) and the ones used by the farmers (open and closed)

	t-test for Equality of Means		
	T	**Df**	**Sig. (2-tailed)**
ln_TOTAL_AF[1](Farm Open vs Farm Closed)	0.309	19.740	0.761
ln_MRL_SA (Farm Open vs Farm Closed)	0.213	19.085	0.834
ln_TOTAL_AF (Farm Open vs Supplier Closed)	-2.535	35.747	0.016**
ln_MRL_SA (Farm Open vs Supplier Closed)	-3.795	32.183	0.001**
ln_TOTAL_AF (Farm Closed vs Supplier Closed)	-3.138	50.773	0.003**
ln_MRL_SA (Farm Closed vs Supplier Closed)	-4.404	42.594	0.000**

T-t statistic, Df-degree of freedom, Sig (p-value) *

[1] * significant at 5%

Table 4.14: Summary of fumonisin contamination in animal feed analysed using Enzyme-linked immunosorbent assay (ELISA)

Type of storage	Mycotoxins	Mean (ppb)	Range (ppb)	Std. Dev	>MRL
Open storage - EF	FB_S	847.69	5270 – 414	1389.33	5(26.3)
Closed storage - EF	FB_S	365	2750 – 120	2600	-
Closed storage - CFS	FB_S	1389.33	619.3 – 50	546.13	-

Ef – emerging farms, cfs – commercial feed supplier*

Represent the samples that tested positive for Aflatoxin analyzed using screening test ELISA. Aflatoxin is present in sampled feeds about 97.7% are positive which are a complaint, there were samples that were detected above the Maximum Tolerated Limits (MTL).

Table 4.15: Represents the samples that tested positive for Aflatoxins B1, B2, G1, G2 analyzed using screening test ELISA

Mycotoxins	n	Positive %	Mean (ppb)	Std. Dev	Range (ppb)	Detection > MTL (%)
Aflatoxins B1, B2, G1, G2	90	88 (97.7)	748.3	173.93	0.75 – 0.56	60(66.7)
Emerging farmers	60	58 (96.6)	326.3	115.71	0.72 – 0.55	29(48.3)
Commercial suppliers	30	30 (100)	422.4	185.2	0.71 – 0.51	19(63.3)

n = total number of samples *

Fumonisin

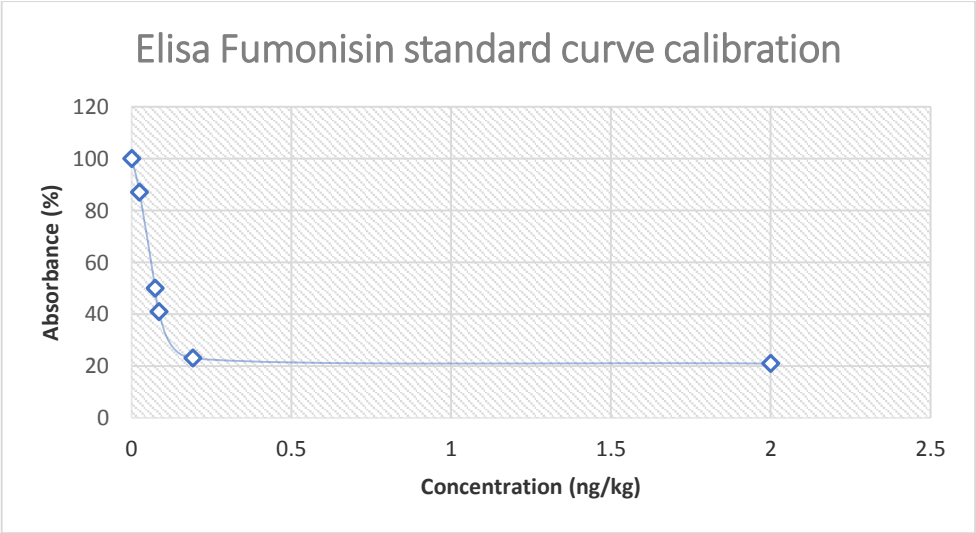


Figure 4.28: Represent the fumonisin standard calibration curve obtained from the $R^2 = 0.9983$ value shows and regression value y = -13.889x + 99.565

Data obtained and analysis of fumonisin on Table 4.16 – 4.17 showed that the differences noted in the preceding table are merely by chance and that the type of storage has no significant impact (p-value > 0.05) on the mean contamination of the samples collected from the farmers.

Table 4.16: Summary of mean differences between storages for Fumonisin

	Type of storage	n	Mean	Std. Deviation	Std. Error Mean
B1_fum	Open storage (Farmers)	15	294.660	317.223	81.907
	Closed storage (Farmers)	14	329.500	561.172	149.979
B2_fum	Open storage (Farmers)	15	50.440	106.916	27.605
	Closed storage (Farmers)	14	23.257	57.261	15.304
Total_Fum	Open storage (Farmers)	15	345.107	324.656	83.826
	Closed storage (Farmers)	14	352.750	609.764	162.966

Table 4.17: Confirm the relationship between the type of storage and the mean mycotoxin contamination using t-test statistics

	t-test for Equality of Means		
	t	**Df**	**Sig. (2-tailed)**
ln_B1_fum (Farm Open vs Farm Closed)	0.307	26.630	0.761
ln_B2_fum (Farm Open vs Farm Closed)	0.572	26.149	0.572
ln_Total_fum (Farm Open vs Farm Closed)	0.486	26.693	0.631

T-t statistic, Df-degree of freedom, Sig (p-value) *

Table 4.18: Represents the samples that tested positive for Fumonisin analyzed using screening test ELISA

Mycotoxins	n	Positive %	Mean (ppb)	Std. Dev	Range (ppb)	Detection > MTL (%)
Fumonisin B_1 & B_2	90	12 (13.3%)	414.89	926.67	5270 - 414	5(5.5)
Emerging farmers	60	5 (8.3)	525.5	1028.146	5270 - 414	-
Commercial suppliers	30	7 (23.3)	193.67	2600	619.3 – 50	-

n = total number of samples *

Ochratoxin A

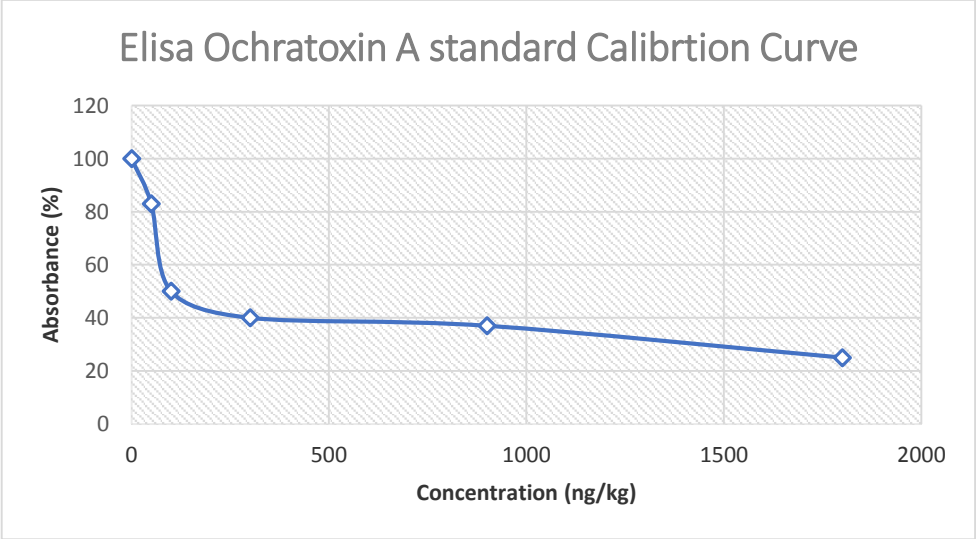


Figure 4.29: the Ochratoxin A standard calibration curve obtained from the R^2 value (= 0.9998) showed regression value y = -0.0053x + 99.926

Table 4.19: Summary of Ochratoxin A contamination in animal feed analysed in open and closed storage facilities of both emerging farmers and commercial suppliers as determined by Enzyme-linked immunosorbent assay (ELISA)

Type of storage	Mycotoxins	Mean (ppb)	Range (ppb)	Std. Dev	<MRL
Open storage - EF	OTA	365.24	492.8 – 4.7	112.62	9 (47.4)
Closed storage - EF	OTA	412.55	541.79 – 4.3	91.96	18 (43.9)
Closed storage - CFS	OTA	2025	558.9 – 38.7	6121.71	22 (55)

Ef – emerging farms, CFS – commercial feed supplier**

Table 4.20: Summary of the differences in the mean contamination of the samples for the different storage systems under study

	Type of storage	n	Mean	Std. Deviation	Std. Error Mean
ELISA_Ocra	**Open storage (Farmers)**	18	352.2	156.606	36.912
	Closed storage (Farmers)	40	400.78	83.371	13.182
ELISA_Ocra	**Open storage (Farmers)**	18	352.2	156.606	36.912
	Closed storage (Suppliers)	32	1460.28	6121.376	1082.117
ELISA_Ocra	**Closed Storage (Farmer)**	42	383.3	113.647	17.536
	Closed Storage (Supplier)	32	1460.28	6121.376	1082.117

Table 4.21: Summary of statistical mean differences between different storage systems for Ochratoxin A

	t-test for Equality of Means		
	t	**Df**	**Sig. (2-tailed)**
ln_ELISA_Ocra (Farm Open vs Farm Closed)	-1.469	18.075	0.159
ln_ELISA_Ocra (Farm Open vs Suppliers Closed)	-1.685	22.837	0.106
ln_ELISA_Ocra (Supplier Open vs Supplier Closed)	-1.375	67.563	0.174

T-t statistic, Df-degree of freedom, Sig (p-value) *

Table 4.22: Represent the samples that tested positive for Ochratoxin A analyzed using ELISA

Mycotoxins	n	Positive (%)	Mean (ppb)	Std. Dev	Range (ppb)	Detection > MTL (%)
Ochratoxin A	90	32 (35.6)	3500.4	365.3	558.9 – 38.9	49 (54.4)
Emerging farmers	60	28 (46.7)	575.7	393.1	541.79 – 4.3	27 (45)
Commercial suppliers	30	4 (13.3)	387	323.2	558.9 – 38.9	22 (73.3)

n= total number of samples *

Zearalenone

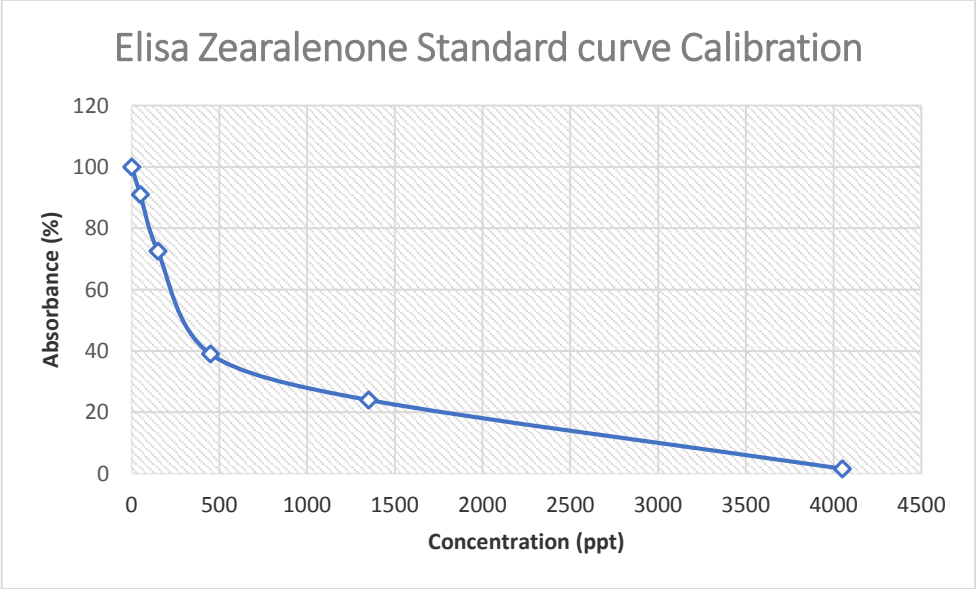


Figure 4.30: Represent the Zearalenone standard calibration curve obtained from the R^2= 0.9995 value showed regression value of y = -0.024x + 98.402

Table 4.24 – 4.25 shows that the storage system has a significant effect (p-value < 0.05) on the mean contamination (ELISA_zea) of the samples collected from the farmers. However, the differences between the mean contamination in the samples collected from the suppliers and that collected from the farmers are due to mere chance and not related to the storage systems. The results in Table 4.23 Revealed that there are differences in the mean contamination of the samples for the different storage systems under study.

Table 4.23: Zearalenone contamination in animal feed analysed in open and closed storage facilities of both emerging farmers and commercial suppliers as determined by Enzyme-linked immunosorbent assay (ELISA)

Type of storage	Mycotoxins	Mean (ppb)	Range (ppb)	Std. Dev	>MRL
Open storage - EF	ZEA	6.96	94.93 – 1.66	16.02	5 (26.3)
Closed storage - EF	ZEA	31.3	142.43 – 3.39	54.09	10 (24.4)
Closed storage - CS	ZEA	7.32	54.94 – 1.14	18.87	-

Ef – emerging farms, CS – commercial feed supplier*

Table 4.24: Zearalenone ELISA results using the T-test analytical method

	Type of storage	N	Mean	Std. Deviation	Std. Error Mean
ELISA_zea	**Open storage (Farmers)**	18	0.82	2.84	0.67
	Closed storage (Farmers)	40	25.20	45.24	7.15
ELISA_zea	**Open storage (Farmers)**	18	0.83	2.84	0.67
	Closed storage (Suppliers)	32	7.09	18.79	3.32
ELISA_zea	**Closed Storage (Farmer)**	40	25.20	45.24	7.15
	Closed Storage (Supplier)	32	7.09	18.79	3.32

Table 4.25: The mean differences observed in table 4.24 are all significant effect (p-value < 0.05)

	t-test for Equality of Means		
	t	**Df**	**Sig. (2-tailed)**
ln_ELISA_zea (Farm Open vs Farm Closed)	-3.494	2.650	.048*[2]
ln_ELISA_zea (Farm Open vs Suppliers Closed)	-2.320	4.190	.078
ln_ELISA_zea (Supplier Open vs Supplier Closed)	1.077	8.094	.313

T-t statistic, Df-degree of freedom, Sig (p-value) *

Table 4.26: Samples that tested positive for Zearalenone analyzed using ELISA.

Mycotoxins	n	Positive %	Mean (ppb)	Std. Dev	Range (ppb)	Detection >MTL (%)
Zearalenone	90	23 (25.6)	15.23	35.29	142.43 – 3.39	18 (20)
Emerging farmers	60	18 (30)	17.05	38.61	142.43 – 3.39	15 (25)
Commercial suppliers	30	5 (16.7)	7.56	19.33	54.94 – 1.14	-

n = total number of samples *

[2] * significant at 5%

4.5 Thin Layer Chromatography results (TLC)

The results for TLC showed high incidence rate of Aflatoxin with a percentage of 82%, 75%, 72% and 63% of Aflatoxin (B_1; B_2; G_1; G_2), with a retention factor of 0.87, 0.81, 0.73, and 0.67, respectively. The TLC quantifying method was used according to (Dutton & Kinsey, 1995) as shown in (Figure 4.31). Table 4.27 illustrates the standard RF and the Sample R_f including the positive sample in percentage (%).

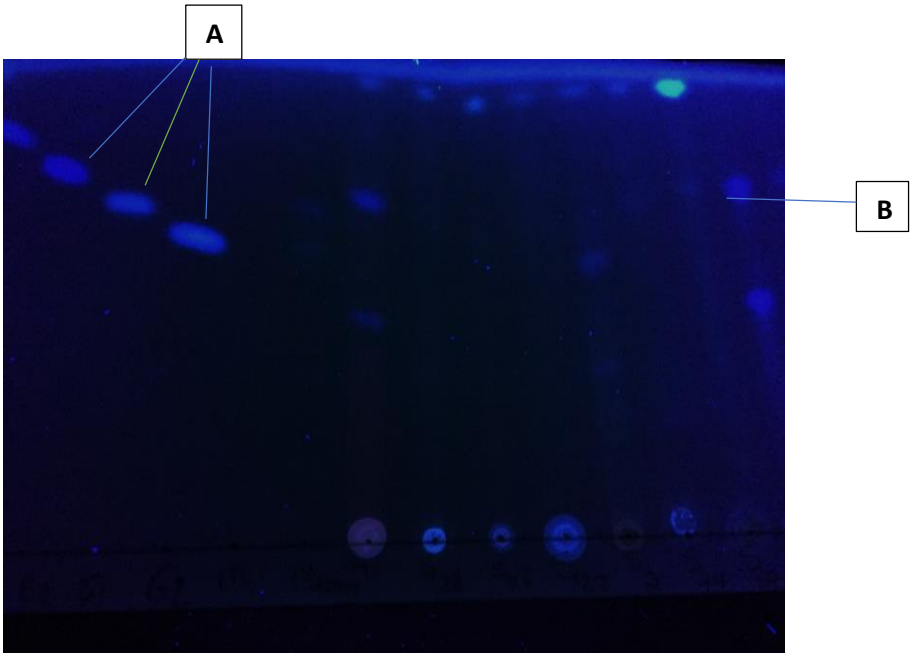


Figure 4.31: A composite picture of TLC plate showing 2.5µl of spotted silica gel paper under fluorescence UV of shortwave 245nm and longwave 365nm, A –Standards (B_1; B_2; G_1; G_2) and B – Sample tested positive for aflatoxin.

Table 4.27: Summarising the Thin Layer Chromatography (TLC) of aflatoxin results found in tested animal feeds whereby the RF value of aflatoxin quantified is < 1.0.

Tested	n	Retention factor (Rf)	The sample tested negative (%)	The sample tested positive (%)
Standards	4	0.69	-	4 (100)
Samples	34	0.89	5 (14.71)	29(85.29)

n = total number of samples *

4.6 High-Performance Liquid Chromatography results

The Immune-Affinity Column (IAC) and Solid Phase Extraction (SPE) were used for extraction and clean up on collected animal feed samples. Samples were analyzed using High-Performance Liquid Chromatography (HPLC) as a confirmatory test. The confirmatory method was coupled with the UltraViolet detector and KOBRA® cell as shown in figures below. Aflatoxin was obtained on HPLC at retention time shown in brackets B_1 (11.4), B_2 (9.2), G_1 (7.9) and G_2 (6.8), for Fumonisin FB1 was between (4.0) and FB2 (7.5) and Zearalenone at (8.0) order standards and samples. The calibration curves Figure of each mycotoxin tested standard obtained with individual R^2 value. Table 4.28 summarises all mycotoxin contamination tested for animal feeds in emerging farms and its commercial supplier, whilst Table 4.29 summarises individual mycotoxin in animal feed tested using HPLC. Below is a table of Maximum Residue Limits (MRL) used in this study (Papiya Mitra Mazumder, 2001)

Table 4.28: A summary of mycotoxin contamination of animal feed analyzed using High-Performance Liquid Chromatography

Mycotoxin analyzed	n	Positive (%)	Mean (ppb)	Range (ppb)	Standard Deviation	Detection > MRL (ppb)
Aflatoxin	100	70 (70)	214.4	0.1- 31.9	174.5	50 (50)
Fumonisin	30	3 (10)	2487.8	2146.1- 0.7	2145.5	-
Zearalenone	100	35 (35)	828.5	788.64 - 0.01	94.29	-

Table 4.29: Summary of aflatoxins contamination in animal fee analysed in open and closed storage facilities of both emerging farmers and commercial suppliers as determined by High-Performance Liquid Chromatography (HPLC).

Type of storage	Mycotoxins	Mean (ppb)	Range (ppb)	Std. Dev
Open storage - EF	AFB_1	0.0	0.0 – 0.0	0.0
Closed storage - EF	AFB_1	0.0	0.0 – 0.0	0.0
Closed storage - CS	AFB_1	0.0	0.0 – 0.0	0.0
Open storage - EF	AFB_2	376.52	0.1-4.59	1099.7
Closed storage - EF	AFB_2	54.73	8.42 – 0.3	241.25
Closed storage - CS	AFB_2	7653.67	89.4 – 1.2	2059.42
Open storage - EF	AFG_1	69.49	23.22 – 1.1	176.79
Closed storage - EF	AFG_1	172.08	43.47 – 1.4	410.36
Closed storage - CS	AFG_1	2113	35.25 – 0.3	1202.92
Open storage - EF	AFG_2	108.62	85.64 – 1.4	176.79
Closed storage - EF	AFG_2	134.47	58.66 – 2.1	174.42
Closed storage - CS	AFG_2	70.80	29.7 – 0.6	280.52
Open storage - EF	Total AFS	554.64	53.36 – 1.1	1267.7
Closed storage - EF	Total AFS	1062.28	98.82 – 3.1	2795.20
Closed storage - CS	Total AFS	9836.47	120.27 – 2.1	26309.9
Open storage - EF	Total MRL	0.55	5.32 – 0.1	1.26
Closed storage - EF	Total MRL	1.06	9.87 – 0.3	2.79
Closed storage - CS	Total MRL	9.83	125.0 – 0.2	26.30

Ef – Emerging farms, CFS – Commercial feed suppliers*

Table 4.30: Summary of fumonisin contamination in animal fee analysed in open and closed storage facilities of both emerging farmers and commercial suppliers as determined by High-Performance Liquid Chromatography (HLPC).

Type of storage	Mycotoxins	Mean (ppb)	Range (ppb)	Std. Dev
Open storage - Ef	FB_1	157.72	413.74 – 0.7	214.51
Closed storage - Ef	FB_1	5.73	14.18 – 4.1	6.24
Closed storage - CS	FB_1	163.45	428.38 – 31.1	220.30
Open storage - Ef	FB_2	314.76	1049.59 – 10.5	367.69
Closed storage - Ef	FB_2	344.61	344.61 – 20.1	31839
Closed storage - CS	FB_2	372.36	1051.78 – 10.1	355.30
Open storage - Ef	FBs total	346.14	1927.5 – 30.6	628.43
Closed storage - Ef	FBs total	25.53	217.76 – 10.2	64.17
Closed storage - CS	FBs total	317.67	2145.31 – 36.1	685.03

Ef – emerging farms, CFS – commercial feed suppliers*

Table 4.31: Summary of zearalenone contamination in animal fee analysed in open and closed storage facilities of both emerging farmers and commercial suppliers as determined by High-Performance Liquid Chromatography (HPLC).

Type of storage	Mycotoxins	Mean (ppb)	Range (ppb)	Std. Dev
Open storage - Ef	ZEA	2.20	661.13 – 0.02	7.91
Closed storage - Ef	ZEA	2.64	578.69 – 0.05	6.43
Closed storage - CS	ZEA	0.16	788.6 – 0.01	0.43

Ef – emerging farms, CS – commercial farms*

Table 4.32: Represent mycotoxin contamination of animal feeds analyzed in emerging farms and commercial supply, determined using HPLC.

Mycotoxin analyzed	n	Positive (%)	Mean(ppb)	Range (ppb)	Std. Dev	>MRL
$AfB_{1=}$ EF	60	-	0.0	0.0 - 0.0	0.0	-
$AfB_{2=}$ EF	60	28 (46.7)	52.9	1.63-1004.2	148.89	12 (20.0)
$AfG_{1=}$ EF	60	29 (48.3)	73.9	0.53-711.9	149.29	14 (23.3)
$AfG_{2=}$ EF	60	25 (41.7)	323.3	1261.2 – 0.83	947.58	22 (36.67)
$AfB_{1=}$ CS	40	-	0.0	0.0 – 0.0	0.0	-
$AfB_{2=}$ CS	40	32 (80.0)	2486.0	1458.8-3.36	5413.54	23 (57.5)
$AfG_{1=}$ CS	40	14 (35.0)	80.3	1881.8 – 0.86	316.28	6 (15.0)
$AfG_{2=}$ CS	40	16 (40.0)	998.7	1468.1 – 0.53	3148.61	12 (30.0)
$FB_{1=}$ EF	15	3 (20.0)	250.9	1926.7 – 29.1	491.89	1 (6.67)
$FB_{2=}$ EF	15	1 (6.67)	21.3	217.5 – 10.2	54.97	-
$FB_{1=}$ CS	15	6 (40.0)	416.1	1927.5 – 30.6	522.26	1 (6.67)
$FB_{2=}$ CS	15	3 (20.0)	55.5	344.6 – 20.1	106.16	-
$ZEA_{=}$ EF	60	16 (26.67)	2.204	661.13 – 0.02	6.43	-
$ZEA_{=}$ CS	40	20 (50.00)	31.601	788.64 – 0.01	0.43	-

Ef = Emerging farm, CFS = Commercial Feed Supplier*

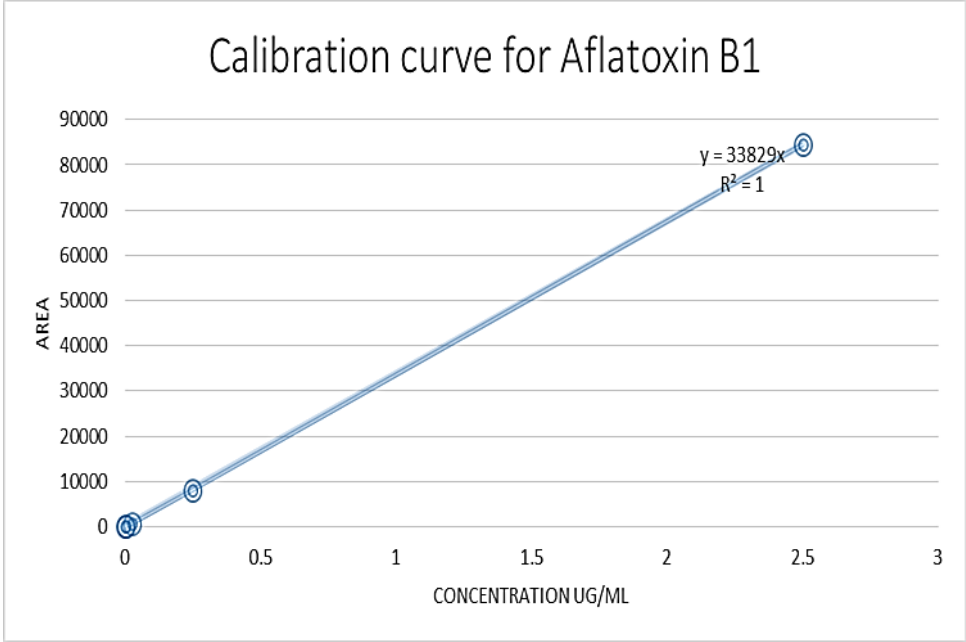


Figure 4.32: Calibration curve of Aflatoxin B_1 standards at 0, 0.3, and 2.5µg/ml at 40 µl injection on High-Performance Liquid Chromatography coupled with an ultraviolet detector and a KOBRA® cell.

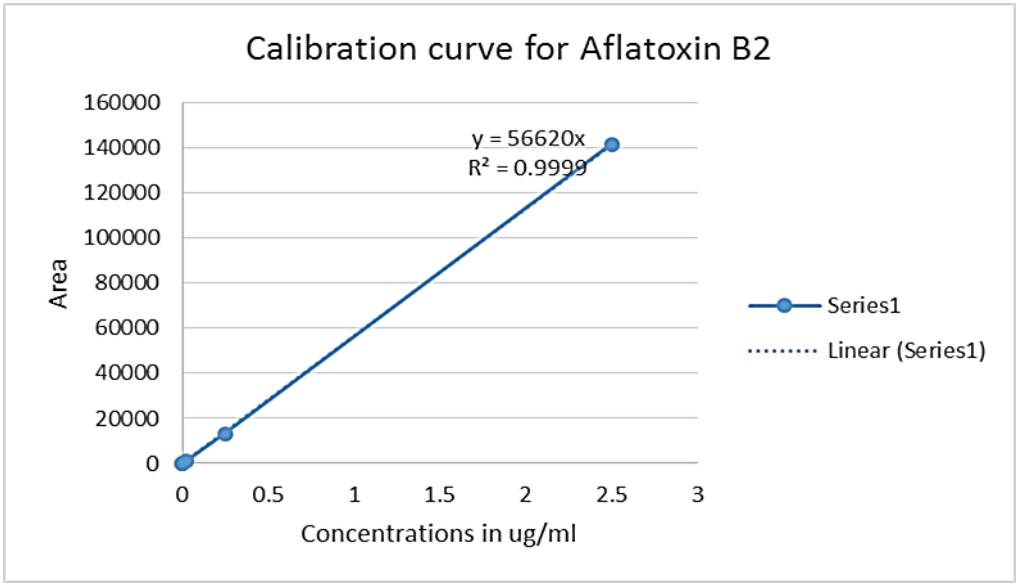


Figure 4.33: Calibration curve of Aflatoxin B_2 standards at 0, 0.3, and 2.5µg/ml at 40 µl injection on High-Performance Liquid Chromatography coupled with an ultraviolet detector and a KOBRA® cell.

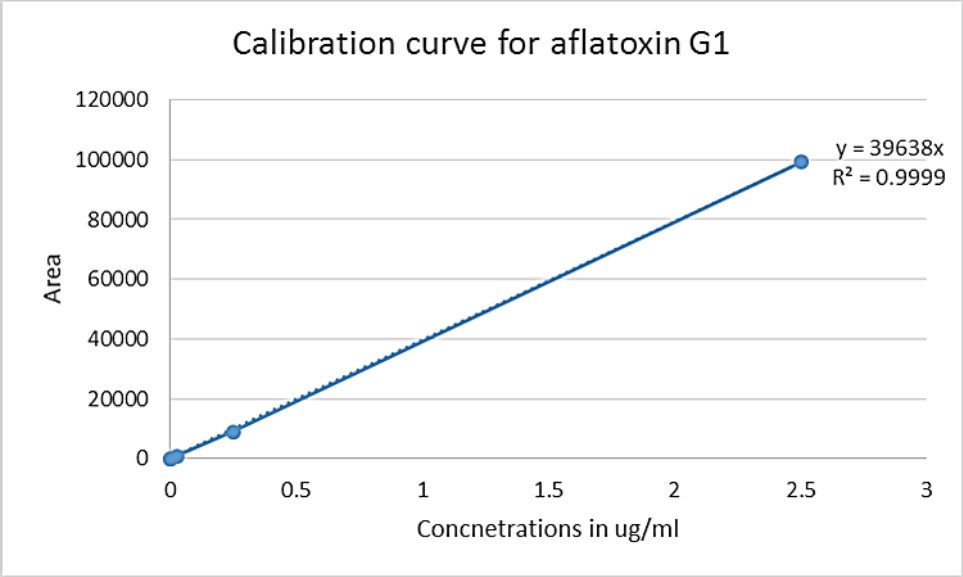


Figure 4.34: Calibration curve of Aflatoxin G_1 standards at 0, 0.3, and 2.5µg/ml at 40 µl injection High-Performance Liquid Chromatography coupled with an ultraviolet detector and a KOBRA® cell.

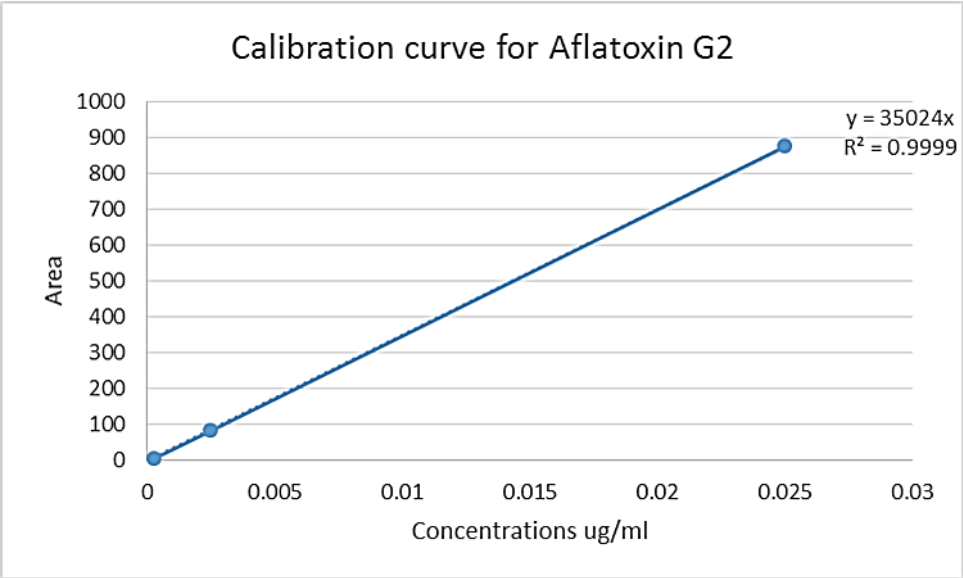


Figure 4.35: Calibration curve of aflatoxin G_2 standards at 0, 0.003, and 0.025µg/ml at 40 µl injection on High-Performance Liquid Chromatography coupled with an ultraviolet detector and KOBRA® cell.

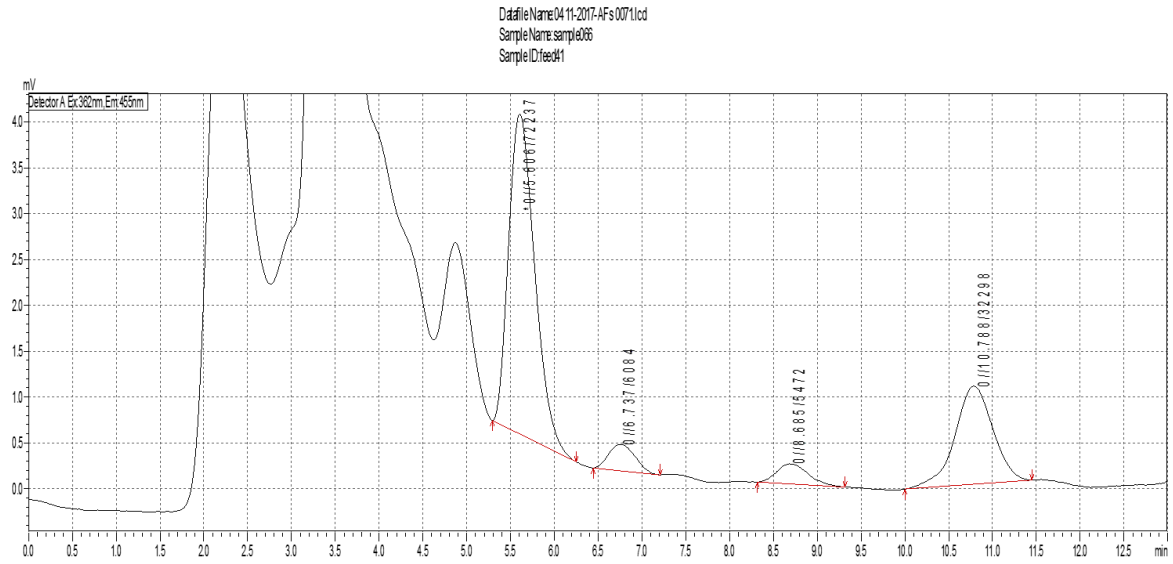


Figure 4.36: Illustration of a chromatogram of sample 41 from emerging farm at 40µl injection contaminated with Aflatoxin $B_1B_2G_1$ and G_2 on High-Performance Liquid Chromatography coupled with an ltraviolet detector and KOBRA® cell.

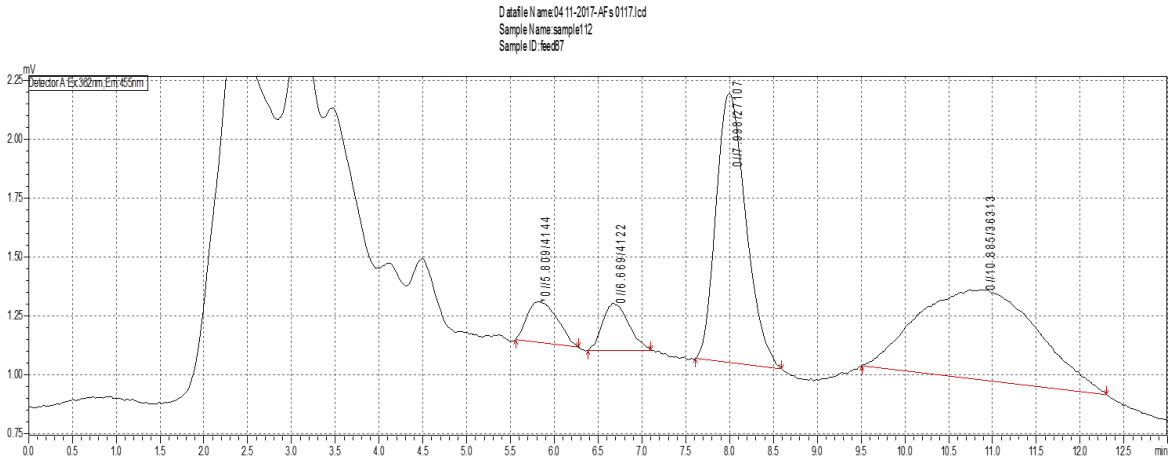


Figure 4.37: Illustration of a chromatography of sample 87 from the commercial supplier at 40µl injection contaminated with Aflatoxins B_1, B_2, G_1 and G_2 on High-Performance Liquid Chromatography coupled with an ultraviolet detector and KOBRA® cell.

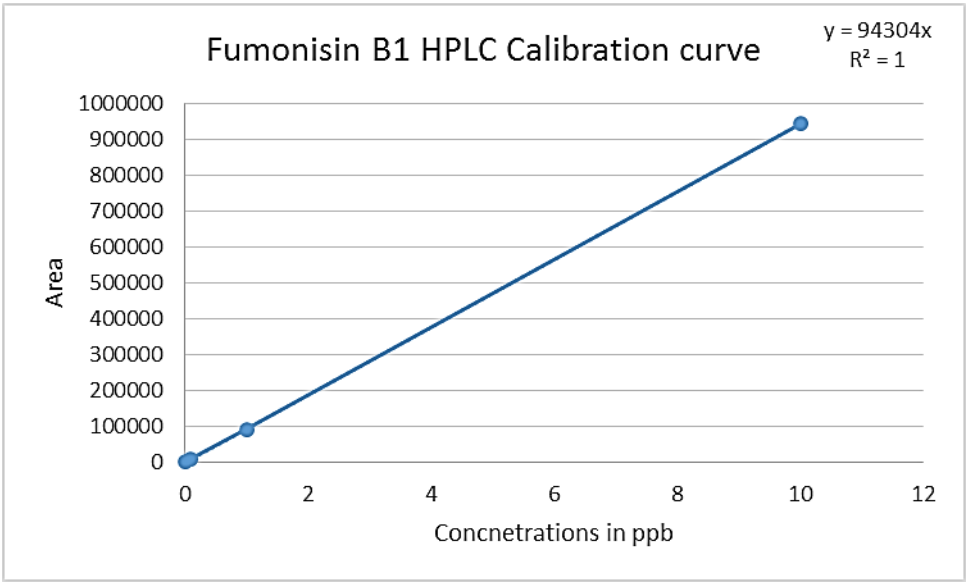


Figure 4.38: Calibration curve of Fumonisin B_1 standards at 0, 1 and 10ppb at 40 µl injection on High-Performance Liquid Chromatography coupled with an ultraviolet detector and KOBRA® cell.

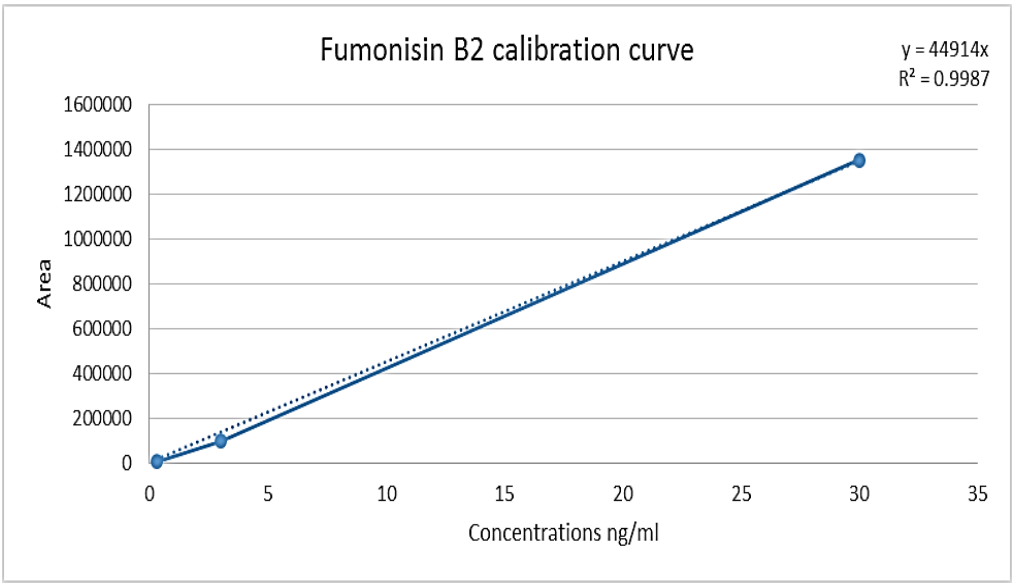


Figure 4.39: Calibration curve of Fumonisin B_1 standards at 0, 3 and 30 ng/ml at 40 µl injection on High-Performance Liquid Chromatography coupled with an ultraviolet detector and a KOBRA® cell.

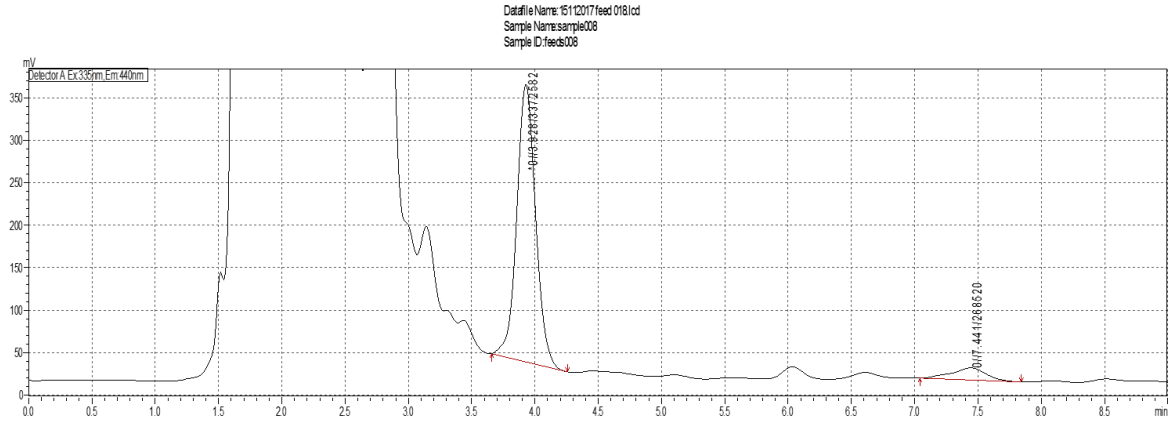


Figure 4.40: Illustration of chromatography on a sample 08 from emerging farm at 40µl injection contaminated with Fumonisins B_1 and B_2 on High-Performance Liquid Chromatography coupled with an ultraviolet detector and KOBRA® cell.

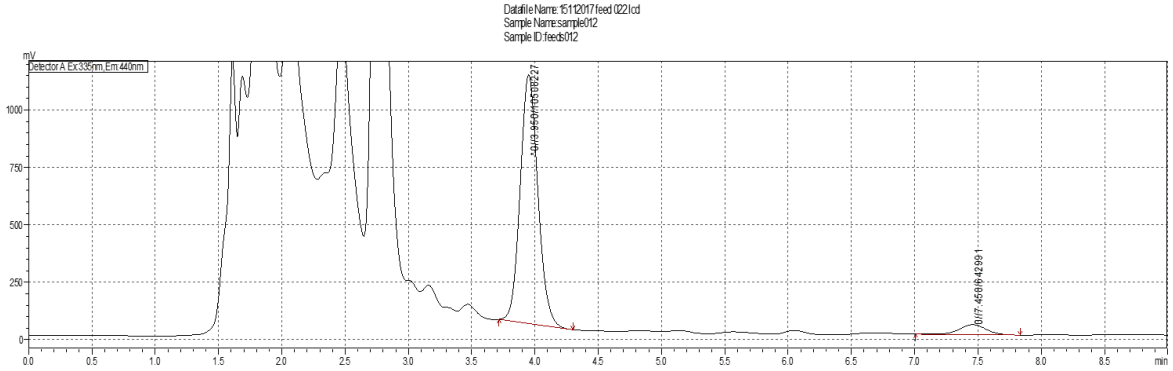


Figure 4.41: Illustration of a chromatogram of sample 68 from the commercial supplier at 40µl injection contaminated with Fumonisins B_1 and B_2 on High-Performance Liquid Chromatography coupled with an ultraviolet detector and KOBRA® cell.

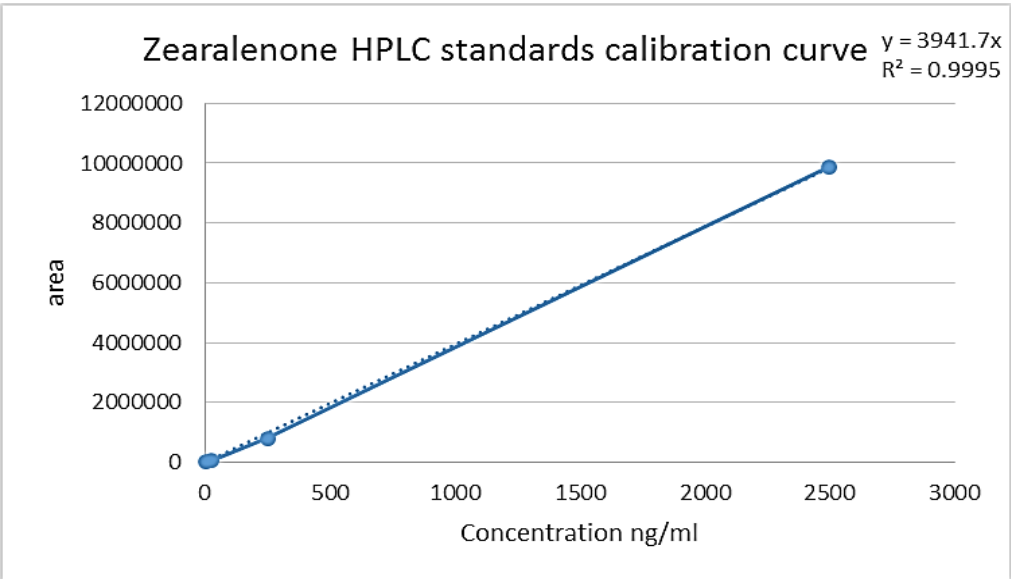


Figure 4.42: Calibration curve of Zearalenone standards at 0, 300 and 2500 ng/ml at 40 µl injection on High-Performance Liquid Chromatography coupled with ultraviolet detector and a KOBRA® cell.

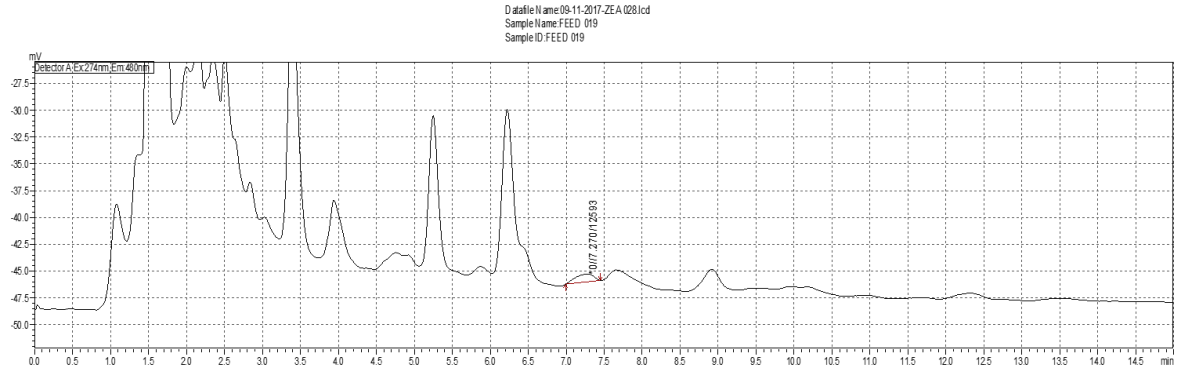


Figure 4.43: Illustration of a chromatogram of feed sample 19 from emerging farm at 40µl injection contaminated with Zearalenone on High-Performance Liquid Chromatography coupled with an ultraviolet detector and KOBRA® cell.

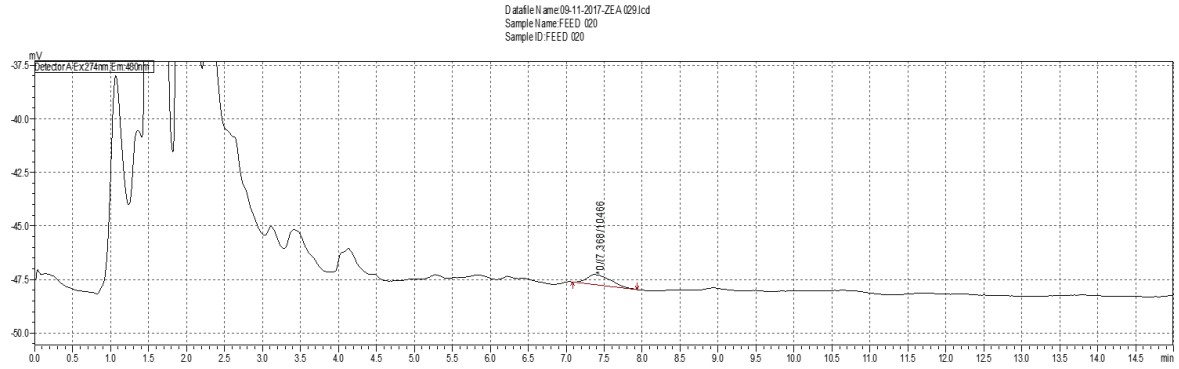


Figure 4.44: Illustration of a chromatography of 89 from the commercial supplier at 40µl injection contaminated with Zearalenone on High-Performance Liquid Chromatography coupled with an ultraviolet detector and KOBRA® cell.

CHAPTER V

DISCUSSION

In this study, interviews were carried out to obtain the view and level of knowledge of farmers and suppliers about the proper way of storing animal feed. Laboratory analyses were also done to measure the quantity of contaminants found in the sampled feeds. The demographic results obtained in this study revealed that 100% of emerging farms in the Ngaka Modiri Molema District, North West province were owned by black male farmers between the age of 31-40 years old, no females was involved, this could be as a result that women in agriculture and rural areas are considered to have one thing in common across regions: they have less access than men to productive resources and opportunities as confirmed by (Cuberes & Teignier, 2014). However, among white farmers, 33.3% of them are female but most of them are in the feed supply industry while 67% of agricultural products are owned by white people. These results agree with the data of Stats SA (2009).

Data on the level of education of emerging farmers revealed that 41.7% attended secondary level, 50% tertiary level and 8.3% postgraduate level, this indicated that there is a radical improvement compares to previous years where black farmers were not educated and could not read nor write. The low level of education amongst farmers was characterized by lack of credit access, absence of innovative production implements needed in-order to increase the yield of the commodity produced and poor entrepreneurial skills needed to make the efforts of the farmers a success as explained by (Bernabe & Vermeulen, 2011)

It was noted that emerging farmers in the study did understand the need to vaccinate their livestock which improved the herd immunity leading to less disease among animals and high productivity (Perry & Randondolph, 1999). It was also noted that most farmers did not have knowledge of fungi and mycotoxins. This has a consequence, lack of proper storage facilities implemented on the farm as well as risk for animals. Farmer's knew about feed contamination, although they do not have much knowledge about mycotoxin contamination of feed. About 100% of emerging farmers are self-employed compared to apartheid-era where they had to be employees, most black people, worked on commercial farms belonging to white farmers to earn a living (Aliber *et al*., 2009).

There is a fundamental improvement in the agricultural sector in South Africa whereby farmers get helped from the Department of Agriculture as well as from State Veterinarians. Emerging farmers around Ngaka Modiri Molema District got 16.7% monthly, 41.7% annually, 25%

sometimes and 16.7% visitations (Figure 4.4). It was also noted that the major challenges faced by emerging farmers versus the commercial ones were that they did not have access to the land for most of them, they are not trained, did not have access to the market so they can sell their animals or animal products. Emerging farmers choose to feed their livestock with mixed feed, amongst other compounds of feed products (Wikipedia, 2017). Mixed feeds are more prone to damage than individual ingredients due to the interaction between different ingredients and because of cross contamination of insects and fungi (Dowd, 1998).

Data collected also revealed that the majority of farmers did not produce feed for animals but obtained from feed suppliers in the area. Feed contamination, in this case, would have been from different sources such as the field obtained from, the processing, the suppliers' storage or the recipient farmer's storage. Contaminants could persist in harvested and stored grain an grow in storage when moisture content becomes favourable (Miller, 2008). This may explain their presence in these analysed samples from both storages of emerging farmers and feed suppliers storage by late harvesting and proper storage (Dutton and Kinsey, 1995)

It was also found in this study that farmers mainly used two types of the storage systems; open and closed storage systems and they preserve their animal feed either in bags or dustbins and their ventilation is by opening windows and garage door, this elucidates the presence of insects in the sampled feedstuff, hence a few feedstuffs were damaged.

It is important to mention that, although some fungi were field fungi, and others were storage fungi, the storage facilities and conditions played an important role in the occurrence of mycotoxins and their producing fungi (Magan *et al* .,2003). (Čonková *et al.*, 2001) and (Atanda *et al.*, 2011) confirm that the production of mycotoxins depends upon numerous external factors (relative humidity, temperature), as well as upon the properties of the substrate (composition, water activity (aw), the degree of contamination) which are mostly the environment in which food and feed are stored. Although the FAO (2011) suggests that feed storage should be provided with ventilation points however, windows or doors are not recommended to avoid rodents entering the store (Science & Development, 1978). Emerging farmers do not store their feed for too long, as they only buy maximum feed for a short period. This might explain why the level of contamination was not so high as compared to the suppliers. In this study, about 50% of farmers stored their feed for about 1-3 weeks and 50% for 1-3 months, as compared to commercial feed suppliers who keep their feed until the expiry date, therefore more fungal contaminants were from feed sampled from the suppliers' storage.

Atanda *et al*. (2011) also confirmed that the storage length is a major factor affecting feed quality in addition to the one mentioned above.

In addition, they also mentioned that extrinsic factors such as climate or intrinsic factors such as fungal strain specificity, strain variation, and instability of toxigenic properties are more difficult to control. There are larger variety of fungi isolated in the closed storage as compared to the open ones, these findings agree with the study of Mwanza *et al* (2012) who also noted that the environment and the type of storage had a significant impact on the occurrence of fungi. In addition, the moisture content played an important role in the survival of fungi and mycotoxins production. This has also been confirmed by Magan *et al* (2007) who found that early (high moisture content in crops) or late (enough to favour Fusarium contamination) harvesting of crops in rural areas and the lack of poor storage facilities characterized by poor ventilation, high temperature and humidity influenced the occurrence of fungi and of the Cfu/g. While Karmas (1980) found that the optimal moisture content for feed should not exceed 12-13% and the relative humidity of air should be under 70%.

This study showed significant differences ($P>0.05$) higher moisture content in feed obtained from closed as compared to open storages (Table 4.2). About 53% of sampled feed from emerging farm closed storage were more contaminated due to the high moisture content in them compared to 30% of open storage feed sampled from emerging farmers, although the commercial suppliers had the highest percentage 100% of the moisture content of feed in them. This might be explained that this was due to poor drying method, the food being stored for a long period of time, the poor structure used for storage is not suitable for storing feeds (e.g. in the garage) (Lacey, 1991).

The study of Lacey (1991) showed that fungal growth occurs between 10 and 40°C, over a pH range of 4 to 8 and aw levels above 0.70, but they can sometimes grow on a very dry surface as well. In this study three major fungal genera were isolated, such contaminants are widely distributed and are always present in stored feed as reciprocate of storage fungi, among which the most important mycotoxin-producing strains were *Aspergillus* spp (54%), *Penicillium* spp (36%) and *Fusarium* spp (10%). Among isolated fungal species, *A. flavus, A. oryzae, A. terreus, A. fumigatus, A. clavatus, A. niger, A. parasisticus, A. nomius, P. verrucosum, P. chrysogenum, P. polonicum, P. rubens, P. brevicompactum* and *F. oxysporum* were the main contaminants (Table 4.5 – 4.7), respectively in feeds obtained from commercial supplier and emerging farmers storage facilities might be explained by the fact that in commercial suppliers

storage usually store a large quantity of grain is in bulk and that produce a condition which is suitable for fungal growth, such increases the concentrations and moisture content (Pitt & Hocking, 1997) and that emerging farmers do not have proper storage facilities to store their feedstuff. This favours humidity, fluctuating temperatures (very hot during the day and very low at night) which favours the growth of these fungi. This is in line with the study done by (Ekwomadu *et al.*, 2017).

The occurrence of *Penicillium* spp. as observed in this study, might be explained by high humidity and moisture content in the crops used for feed production due to early harvesting (Magan et al., 2007) intended to avoid scavenging animals and robbers to spoil (Mwanza *et al*., 2012) and removal of crops early results in crops with high moisture content which will take longer to dry and make them prone to fungal contamination such as saprophytes, and pathogens such as *Fusarium* spp. (Pitt and Hocking, 1997; Sweeney and Dobson, 1998). While, the isolation of *Fusarium* spp, although it was not of high occurrence might be explained by the late harvesting being favourable for their growth (Sweeney and Dobson, 1998).

In addition, the drying facilities as well as the type of storage, mainly open, make it difficult to control factors such high temperature during the day and high humidity at night (Mwanza, 2007) result in favourable conditions for contamination with storage fungi such as *Aspergillus* and *Penicillium* and *Penicillium* spp. are mainly isolated in temperate regions (Pitt and Hocking, 1997) and other *Aspergillus* spp. (Klich, 2007) are also known to appear in temperate regions such as Europe and North America, might also be justified by the climate changes observed globally and in the region in recent years characterized by high rainfall causing high humidity and changes in temperatures (unusual low) in summer and increasingly low temperatures in winters recorded recently in South Africa in particular and in Africa in general.

It is important to mention that fungal contamination in the feed will not only depend on the type of storage but depend also on the quality of feed as contamination can occur I the field, during harvesting, processing or storage. All these factors need to be taken into consideration while analysing the cause of feed contamination.

Results obtained in this study also showed that some isolated fungal strains could not be identified with certainty to the species level. This was the case with the fungi which were allocated the accession numbers MG 647839 (fungal spp. at 99%); MG 647860, MG 647852 and MG 647866 (*Aspergillus* spp. at 99%) and MG 647874 (*Aspergillus* spp.). These can also be explained by the environmental changes characterized by global warming where new strains

are isolated, and mutations are occurring, or they were just different organisms. The isolation of uncommon fungal strains such as unusual *Penicillium* spp. or unconfirmed *Aspergillus* spp. more common in temperate and cold regions or practically unknown in the tropics have as consequence the appearance and production of new mycotoxins not always found in the region and lead to a new clinical and pathological symptomatic appearance of new mycotoxins with possible repercussions on animals and humans. More investigations to molecularly characterise these fungi and their production of mycotoxin capacity and their toxigenicity are needed.

In the review of Mwanza (2012) which confirmed that the highest potential risks for animal food safety comes from chemical contamination which is regarded as the most common risk to produce mycotoxins, which are formed naturally within grains. Mycotoxin detection and quantification was done using ELISA and clean-up method SPE. The values obtained were lower compared to the Maximum Tolerated Limits (MTLs) in ppb according to RSA (Mazumder & Sasmal, 2001).

Data obtained revealed that there were no significant difference between open and closed storages. However slight differences were observed between closed and open storage with closed ones having higher concentrations of mycotoxins. This can be explained mainly by the differences in the type of feed samples used by farmers, also the storage times were not consistent from all farmer groups. This was the major challenge and limitation which this study faced.

To obtain proper data, there is a need for consistency regarding the type of feed to be sampled and time of storage. The differences observed between closed and open storages might be explained by the fact that in closed environments there is elevated temperature and humidity favouring fungal growth and mycotoxin production as compare the open storages.

The occurrence of these fungal species is a cause for concern because most of the species isolated are producers of major mycotoxins such as aflatoxins, fumonisin, ochratoxin A, and zearalenone leading to mycotoxin contamination, a serious food safety issue world-wide (FAO, 2013). Thus these fungi have an enormous impact on animals as it affects their productivity and performance, in addiction such effects can leads to death in animal and humans. In this study, Aflatoxin was the most predominant isolate found in all selected storages both (closed and open) Table 4.11 although high mean concentration 632.03 - 618.91 ppb were conducted from closed storages from emerging farm and feed commercial suppliers, respectively. These means were beyond the acceptable regulated levels in South Africa and in the world. The presence of these aflatoxins correlates with the occurrence of aflatoxin producing strains isolated in feeds, mainly *A. flavus* the B-type and *A. parasiticus* that was also isolated in this

study produce both B-type and G-type Afs (AFG1 and AFG2). The results of this study are in line with the study done by (Marin *et al.*, 2013). This study analysed the presence of AFs showing Aflatoxin B2 was the most dominant, followed by AFG2, AFG1 and AFB1. As said in chapter 2, aflatoxins as other mycotoxins are challenging to extract and detect, very low levels can do harm, and the findings of this study are in line with other studies done by (Mwanza *et al*., 2014). In this study, some of the samples were having more AFB2 than AFB1, this can be explained through the extraction methods or techniques used by the technician or student which can affect the recovery, or by the degree of AFB1, AFG 1 and 2 gene regulations which are always influenced by several environmental factors such as climatic conditions. This is shown also in Table 4.29 which revealed that AFB1 was not present, although other metabolized products were detected, this might be due the environmental effects and climate change (Grío *et al.*, 2010). The presence of these aflatoxins can also be explained through improper handling of feed, both during harvesting and storage (Zaki *et al.,* 2012). *Fusarium* spp. were the least mycotoxin producers both in terms of prevalence and contamination levels in contrast to other mycotoxins in this study. The contamination levels of Fumonisin in this study Table 4.30 revealed that there were high concentration of FB2 compared to FB1 in both storages (closed and open) of emerging farms and closed storage of feed commercial supplier, with mean concentration of 25.53 ppb - 346.14 ppb and 317.67 ppb respectively, this explains the presence F. *oxysporum* isolates. It is worth mentioning that these findings could be due to some fungal strains possibly lost amongst others, especially when concentration levels of other fungal strains were much higher (Njobeh *et al*., 2009). The levels of Zearalenone in this study as presented in Table 4.31 revealed low mean concentrations of 2.20 – 2.64 ppb and 0.16 ppb in emerging farm storages (Closed and open respectively) and commercial feed suppliers closed storage, respectively. Lower levels of Zearalenone mycotoxin are common due to conceivable biotransformation of the conjugated mycotoxin from the parent mycotoxin (Stoev *et al.*, 2010). In most studies done in South Africa, ZEN is conveyed in lower concentrations, which makes this mycotoxin less problematic (DAFF, 2018).The presence of aflatoxins and ochratoxin A in this study correlate with finding on fungi isolation which showed the occurrence of *Aspergillus* species such as *A. fumigatus, A. niger* and *A. flavus and Penicillium* spp. which are known to be producers of these mycotoxins (Stoev, 2010).

The results above were confirmed using T-tests was to confirm the relationship between the type of storage and the mean fungal contamination of the proceeding methodological tests done for Aflatoxin, Fumonisin, Zearalenone, and Ochratoxin were present but not in high quantities

compared to the study done by Mwanza, (2012) which had a high concentration of contaminants found in animal feeds. The presence of these mycotoxins in analysed samples can be explained by the isolated fungi (*Aspergillus*, *Fusarium* and *Penicillium*) species in feed samples which would produce these mycotoxins.

The presence of Zearalenone in the analysed samples correlated with the fungal analysis findings done by (Sydenham *et al.*, 1990). These results are in line with the ones reported by Mwanza *et al*. (2012) who in their study on feed samples collected from Limpopo and Mpumalanga also reported the occurrence of Fumonisin, Zearalenone, and aflatoxins as major contaminants of animal feeds used in rural areas. However, Mwanza's results were significantly higher than the values obtained in this study. The differences would be explained by the environmental factors such as high rainfalls, temperatures and humidity recorded in Limpopo and Mpumalanga as compared to the North West Province know to be hot but dry. These mostly dry and hot conditions do not favour much production of fungi and mycotoxins.

The results obtained for mycotoxins showed high standard deviations which was indicative of the variation of mycotoxins concentrations in feed. This was affected by the variability of feed collected and also explains the fact that mycotoxins do not contaminated feed homogenously. In addition, there was a significant difference between data obtained from commercial suppliers and emerging farmers. The difference could be explained by the fact that commercial suppliers use closed storages without proper monitoring of the environment (temperature and humidity) but also because they store large quantities of feed which takes time to finish. Whereas with emerging farmers most of them do not have significant financial means to buy in bulk, only buy feed for short periods, however whether the storage of feed is long or of short period, if the conditions become favourable they allow fungi and mycotoxins to increase.

Therefore, it is essential to mention that the impacts of climatic variation influence the occurrence of fungi and mycotoxins. This is confirmed by Medina (2015), who confirms that mycotoxin production may be significantly increased under climate change scenarios. In addition, Patterson et al (2018) also confirmed that mycotoxins are among the major foodborne risks that are most susceptible to climatic change. The changes on food and feed safety are issues which need a lot of attention as this might also explain the presence of these fungi in the analysed feedstuff form areas where no report of these mycotoxins had previously been reported.

CHAPTER VI

CONCLUSION AND RECOMMENDATION

The main objective of the study was to assess the effects of storage facility type on fungal contamination and mycotoxins accumulation in animal feed collected from emerging famers and feed commercial suppliers. To reach the above mentioned objective, a questionnaire was conducted and it was found from the survey that emerging farmers did not have knowledge of mycotoxins and fungi and their impacts on feed quality. Hence the study aimed to inform farmers and feed commercial suppliers about the effect of storage on toxin production and current toxin levels observed. In this study it was evident that the type of storage in which feeds are kept, had an impact although the influence was not that of difference. The development of storage fungi from analysed storage was influenced by a number of things which included moisture content, temperature and the length of the storage of the animal feed (Egbuta *et al.*, 2017). Thus, this can also be hypothetically explained that the same feeds that are kept in either closed or open storage are likely to contain the same mycotoxins (Shephard *et al.*, 2013). Thus, feedstuff must be kept free of contamination and safe for animal consumption to maintain healthy and safe animal products. Control strategies should be implemented firmly during the post-harvest should include drying and proper storage conditions (Degen, 2011).

In this study closed commercial feed suppliers' storages were highly contaminated due to the prolonged storage of feed, lack of temperature regulation and ignorance, whilst the positive results obtained from samples from emerging farms (closed & open) could be due to poor storage facilities, improper handling conditions and transportation might be some of the factors contributing to feed contamination (Sweetney & Dobson, 1998). In addition, the study showed that there was a significant difference between the closed and open storage regarding fungi and mycotoxin contamination. Due to the limitation regarding feed types, samples that were collected which were not the same for all farms, the study was of a limited capacity and cannot give a firm conclusion regarding the best storage system.

However, the study recommends that there is a need for regular training and information sessions which will help farmers to reduce the risk of fungal and mycotoxins contamination in feed by controlling the environments of closed storages. Furthermore, training should be provided to all farmers and feed suppliers to increase their knowledge about the importance of storing feed properly. Although not much can be done to prevent or reduce field fungi because this microbial contaminant invades the seeds before harvest. However, the following recommendations should help prevent fungal problems or minimize damage from storage fungi

in stored animal feeds (Magan *et al.*, 2011); clean all feedstuff that is going into the storage by removing all foreign materials.

- Clean all handling equipment during harvesting.
- Clean bins, bags, and all feed packaging materials.
- Check stored feedstuff on a regular basis.
- Maintain low moisture and cool temperature.
- Protect feedstuff from insect and mite damage.
- Allow air to come inside the storage area to equalize aeration and maintain a good ventilation.

REFERENCES

1. Afshar, P., Shokrzadeh, M., Kalhori, S., Babaee, Z. & Saravi, S.S. 2013. Occurrence of Ochratoxin A and Aflatoxin M1 in human breast milk in Sari, Iran. *Food Control*, 31(2):525-529.
2. Akande, K., Abubakar, M., Adegbola, T. & Bogoro, S. 2006. Nutritional and health implications of mycotoxins in animal feeds: a review. *Pakistan Journal of Nutrition*, 5(5):398-403.
3. Atanda, O., Ogunrinu, M. & Olorunfemi, F. 2011. A neutral red desiccated coconut agar for rapid detection of aflatoxigenic fungi and visual determination of aflatoxins. *World Mycotoxin Journal*, 4(2):147-155.
4. R. Russell, M.Paterson[a]ArmandoVenâncio[a]NelsonLima[a]MichèleGuilloux-Bénatier[b]SandrineRousseaux[b], Predominant mycotoxins, mycotoxigenic fungi and climate change related to wine. Food Research International, Volume 103, January 2018, Pages 478-491
5. ÁngelMedina, Alicia Rodríguez, NareshMagan, Applied Mycology Group, AgriFood Theme, Cranfield University, Cranfield, Bedford MK43 0AL, UK, and Climate change and mycotoxigenic fungi: impacts on mycotoxin production, Available online 19 November 2015.
6. Aziz, N., Farag, S., Mousa, L. & Abo-Zaid, M. 1998. Comparative antibacterial and antifungal effects of some phenolic compounds. *Microbios*, 93(374):43-54.
7. Bandyopadhyay, R., Kumar, M. & Leslie, J.F. 2007. Relative severity of aflatoxin contamination of cereal crops in West Africa. *Food additives and contaminants*, 24(10):1109-1114.
8. Bankole, S. & Adebanjo, A. 2003. Mycotoxins in food in West Africa: current situation and possibilities of controlling it. *African Journal of Biotechnology*, 2(9):254-263.
9. Benhin, J.K. 2006. Climate change and South African agriculture: Impacts and adaptation options.
10. Bernabe, E. & Vermeulen, H. 2011. Rural Supermarkets in South Africa. *Reconnecting Markets: Innovative Global Practices in Connecting Small-scale Producers with Dynamic Food Markets*:17.
11. Binder, E., Tan, L., Chin, L., Handl, J. & Richard, J. 2007. Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. *Animal feed science and technology*, 137(3):265-282.
12. Blignaut, J., Ueckermann, L. & Aronson, J. 2009. Agriculture production's sensitivity to changes in climate in South Africa. *South African Journal of Science*, 105(1-2):61-68.
13. Bohra, N. & Purohit, D. 2003. Fungal toxicity with special reference to mycotoxins. *Journal of Environmental biology*, 24(3):213-221.
14. Boland, M.J., Rae, A.N., Vereijken, J.M., Meuwissen, M.P., Fischer, A.R., van Boekel, M.A., Rutherfurd, S.M., Gruppen, H., Moughan, P.J. & Hendriks, W.H. 2013. The future supply of animal-derived protein for human consumption. *Trends in Food Science & Technology*, 29(1):62-73.
15. Botlhoko, G. & Oladele, O. 2013. Factors affecting farmers participation in agricultural projects in Ngaka Modiri Molema District North West Province, South Africa. *Journal of Human Ecology*, 41(3):201-206.
16. Bryden, W.L. 2012. Mycotoxin contamination of the feed supply chain: Implications for animal productivity and feed security. *Animal feed science and technology*, 173(1):134-158.
17. Bueno, D., Muñoz, R. & Marty, J.-L. 2013. Common Methods to Detect Mycotoxins: A Review with Particular Emphasis on Electrochemical Detection: Univerzita Pardubice.
18. Cardon, B.P., Stanley, E., Pistor, W. & Nesbitt, J. 1951. The use of salt as a regulator of supplemental feed intake and its effect on the health of range livestock: College of Agriculture, University of Arizona (Tucson, AZ).

19. Chattha, e.a. 2015. NEW WHEAT STORAGE STRUCTURES AND THEIR EFFECTS ON GRAIN QUALITY IN SINDH, PAKISTAN.
20. Choudhary, A.K. & Kumari, P. 2010. Management of mycotoxin contamination in preharvest and post harvest crops: present status and future prospects. *Journal of Phytology*, 2(7).
21. Chu, F.S. 1996. Recent studies on immunoassays for mycotoxins: ACS Publications.
22. Coblentz, W. 2006. Baled silage for livestock. *Popular Publication*, 7(3):13-18.
23. Čonková, E., Laciaková, A., Pástorová, B., Seidel, H. & Kováč, G. 2001. The effect of zearalenone on some enzymatic parameters in rabbits. *Toxicology Letters*, 121(3):145-149.
24. Coulombe, R.A. 1993. Biological Action of Mycotoxins1. *Journal of dairy science*, 76(3):880-891.
25. Crossan, A., Sanchez-Bayo, F., Kennedy, I. & Bodnaruk, K. 2006. Risk of pesticide contamination in cotton seed and livestock. *The Australian Cottongrower*.
26. Cuberes, D. & Teignier, M. 2014. Gender inequality and economic growth: A critical review. *Journal of International Development*, 26(2):260-276.
27. Current Opinion in Food Science, Volume 5, October 2015, Pages 99-104
28. Daly-Koziel, K. & Walters, J. 2012. Solid Fats and Added Sugars (SoFAS): Know the Limits.
29. Daya-Khilosia, L. 2011. A surveillance study of mycotoxins in the South African industry with specific reference to aflatoxin B_1 in feed and aflatoxin M_1 in farm gate and selected commercially available dairy milk. University of Johannesburg.
30. De Saeger, S., Sibanda, L. & Van Peteghem, C. 2003. Analysis of zearalenone and α-zearalenol in animal feed using high-performance liquid chromatography. *Analytica Chimica Acta*, 487(2):137-143.
31. Diekman, M.A. & Green, M.L. 1992. Mycotoxins and reproduction in domestic livestock. *Journal of animal science*, 70(5):1615-1627.
32. Dowd, P.F. 1998. Involvement of arthropods in the establishment of mycotoxigenic fungi under field conditions: Marcel Dekker: New York.
33. Dutton, M.F. & Kinsey, A. 1995. Occurrence of mycotoxins in cereals and animal feedstuffs in Natal, South Africa 1994. *Mycopathologia*, 131(1):31-36.
34. Egbuta, M.A., Mwanza, M. & Babalola, O.O. 2017. Health Risks Associated with Exposure to Filamentous Fungi. *International journal of environmental research and public health*, 14(7):719.
35. Egbuta, M.A., Mwanza, M., Njobeh, P.B., Phoku, J.Z., Chilaka, C.A. & Dutton, M.F. 2015. Isolation of Filamentous Fungi Species Contaminating Some Nigerian Food Commodities. *Journal of Food Research*, 4(1):38.
36. Ekwomadu, T.I., Gopane, R.E. & Mwanza, M. 2017. Occurrence of filamentous fungi in maize destined for human consumption in South Africa. *Food Science & Nutrition*.
37. Fageria, N.K., Baligar, V.C. & Jones, C.A. 2010. Growth and mineral nutrition of field crops: CRC Press.
38. Fandohan, P., Gnonlonfin, B., Hell, K., Marasas, W. & Wingfield, M. 2006. Impact of indigenous storage systems and insect infestation on the contamination of maize with fumonisins. *African Journal of Biotechnology*, 5(7):546-552.
39. Feedlot, B.m.i. 2011. FAD PReP.
40. Fink-Gremmels, J. 2008. The role of mycotoxins in the health and performance of dairy cows. *The Veterinary Journal*, 176(1):84-92.
41. Fink-Grernmels, J. 1999. Mycotoxins: their implications for human and animal health. *Veterinary Quarterly*, 21(4):115-120.
42. Galvano, F., Piva, A., Ritieni, A. & Galvano, G. 2001. Dietary strategies to counteract the effects of mycotoxins: a review. *Journal of Food Protection*, 64(1):120-131.
43. Gelderblom, W., Jaskiewicz, K., Marasas, W., Thiel, P., Horak, R., Vleggaar, R. & Kriek, N. 1988. Fumonisins--novel mycotoxins with cancer-promoting activity produced by Fusarium moniliforme. *Applied and environmental microbiology*, 54(7):1806-1811.
44. Ghiasian, S. & Maghsood, A. 2011. Occurrence of aflatoxigenic fungi in cow feeds during the summer and winter season in Hamadan, Iran. *African Journal of Microbiology Research*, 5(5):516-521.

45. Gorman, D. & Kang, M. 1991. Preharvest aflatoxin contamination in maize: resistance and genetics. *Plant Breeding*, 107(1):1-10.
46. Gotlieb, A. 2002. Mycotoxins in silage: a silent loss in profits. *The Vermont Crops Soils Home Page, http://pss. uvm. edu/vtcrops/Articles/Mycotoxart. htm (consultado en 30-10*.
47. Grío, S.J.L., Frenich, A.G., Vidal, J.L.M. & Romero-González, R. 2010. Determination of aflatoxins B1, B2, G1, G2 and ochratoxin A in animal feed by ultra high-performance liquid chromatography–tandem mass spectrometry. *Journal of separation science*, 33(4-5):502-508.
48. Heathcote, J.G. & Hibbert, J. 1978. Aflatoxins: chemical and biological aspects: Elsevier Scientific Publishing Co.
49. Heidtmann-Bemvenuti, R., Mendes, G., Scaglioni, P., Badiale-Furlong, E. & Souza-Soares, L. 2011. Biochemistry and metabolism of mycotoxins: A review. *African Journal of Food Science*, 5(16):861-869.
50. Hell, K., Cardwell, K., Setamou, M. & Poehling, H.-M. 2000. The influence of storage practices on aflatoxin contamination in maize in four agroecological zones of Benin, West Africa. *Journal of Stored Products Research*, 36(4):365-382.
51. Hsieh, D. 1990. Health risks posed by mycotoxins in foods. *Toxicological Research*, 6(2):159-166.
52. Hussein, H.S. & Brasel, J.M. 2001. Toxicity, metabolism, and impact of mycotoxins on humans and animals. *Toxicology*, 167(2):101-134.
53. Iheanacho, H.E., Njobeh, P.B., Dutton, F.M., Steenkamp, P.A., Steenkamp, L., Mthombeni, J.Q., Daru, B.H. & Makun, A.H. 2014. Morphological and molecular identification of filamentous Aspergillus flavus and Aspergillus parasiticus isolated from compound feeds in South Africa. *Food microbiology*, 44:180-184.
54. Jayas, D.S. & White, N.D. 2003. Storage and drying of grain in Canada: low cost approaches. *Food Control*, 14(4):255-261.
55. Jenkins, G.R., Tolleson, W.H., Newkirk, D.K., Roberts, D.W., Rowland, K.L., Saheki, T., Kobayashi, K., Howard, P.C. & Jr, W.B.M. 2000. Identification of fumonisin B1 as an inhibitor of argininosuccinate synthetase using fumonisin affinity chromatography and in vitro kinetic studies. *J BIOCHEM*, 14(6).
56. Kabak, B. 2009. Prevention and management of mycotoxins in food and feed. Mycotoxins in food, feed and bioweapons. Springer. p. 201-227).
57. Kabak, B., Dobson, A.D. & Var, I.l. 2006. Strategies to prevent mycotoxin contamination of food and animal feed: a review. *Critical reviews in food science and nutrition*, 46(8):593-619.
58. Kan, C. & Meijer, G. 2007. The risk of contamination of food with toxic substances present in animal feed. *Animal feed science and technology*, 133(1):84-108.
59. Khilosia, L.D. 2011. A Surveillance Study of Mycotoxins in the South African Industry with Specific Reference to Aflatoxin B1 in Feed and Aflatoxin M1 in Farm Gate and Selected Commercially Available Dairy Milk. University of Johannesburg.
60. Klich, M. & Pitt, J. 1988. Differentiation of Aspergillus flavus from A. parasiticus and other closely related species. *Transactions of the British Mycological Society*, 91(1):99-108.
61. Klich, M.A. 2002. Indentification of common Aspergillus species: Centraalbureau voor Schimmelcultures.
62. Klich, M.A. 2007. Environmental and developmental factors influencing aflatoxin production by Aspergillus flavus and Aspergillus parasiticus. *Mycoscience*, 48(2):71-80.
63. Krska, R. & Molinelli, A. 2009. Rapid test strips for analysis of mycotoxins in food and feed. *Analytical and bioanalytical Chemistry*, 393(1):67-71.
64. Kumar, S., Stecher, G. & Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular biology and evolution*, 33(7):1870-1874.
65. Lancova, K., Hajslova, J., Poustka, J., Krplova, A., Zachariasova, M., Dostálek, P. & Sachambula, L. 2008. Transfer of Fusarium mycotoxins and 'masked'deoxynivalenol (deoxynivalenol-3-glucoside) from field barley through malt to beer. *Food additives and contaminants*, 25(6):732-744.
66. Lawrence, M.G. 2005. The relationship between relative humidity and the dewpoint temperature in moist air: A simple conversion and applications. *Bulletin of the American Meteorological Society*, 86(2):225-233.

67. Lin, L., Zhang, J., Wang, P., Wang, Y. & Chen, J. 1998. Thin-layer chromatography of mycotoxins and comparison with other chromatographic methods. *Journal of chromatography A*, 815(1):3-20.
68. Magan, N., Medina, A. & Aldred, D. 2011. Possible climate-change effects on mycotoxin contamination of food crops pre-and postharvest. *Plant Pathology*, 60(1):150-163.
69. Malaker, P., Mian, I., Bhuiyan, K., Akanda, A. & Reza, M. 2008. Effect of storage containers and time on seed quality of wheat. *Bangladesh Journal of Agricultural Research*, 33(3):469-477.
70. Malone, B.R., Humphrey, C.W., Romer, T.R. & Richard, J.L. 1998. One-step solid-phase extraction cleanup and fluorometric analysis of deoxynivalenol in grains. *Journal of AOAC International*, 81(2):448-452.
71. Marin, S., Ramos, A., Cano-Sancho, G. & Sanchis, V. 2013. Mycotoxins: Occurrence, toxicology, and exposure assessment. *Food and Chemical Toxicology*, 60:218-237.
72. Mazumder, P.M. & Sasmal, D. 2001. Mycotoxins–limits and regulations. *Ancient science of life*, 20(3):1.
73. McLean, M. & Dutton, M.F. 1995. Cellular interactions and metabolism of aflatoxin: an update. *Pharmacology & therapeutics*, 65(2):163-192.
74. Medina, A., Rodriguez, A. & Magan, N. 2014. Effect of climate change on Aspergillus flavus and aflatoxin B1 production. *Frontiers in Microbiology*, 5:348.
75. Méndez, A. & Moreno, E. 2009. Las micotoxinas, contaminantes naturales de los alimentos. *Revista Ciencia*, 61:1-7.
76. Milićević, D.R., Škrinjar, M. & Baltić, T. 2010. Real and perceived risks for mycotoxin contamination in foods and feeds: challenges for food safety control. *Toxins*, 2(4):572-592.
77. Morgan, S.E. 1989. Gossypol as a toxicant in livestock. *Veterinary Clinics of North America: Food Animal Practice*, 5(2):251-262.
78. Munkvold, G.P. 2003a. Cultural and genetic approaches to managing mycotoxins in maize. *Annual review of phytopathology*, 41(1):99-116.
79. Munkvold, G.P. 2003b. Epidemiology of Fusarium diseases and their mycotoxins in maize ears. Epidemiology of Mycotoxin Producing Fungi. Springer. p. 705-713).
80. Murphy, P.A., Rice, L.G. & Ross, P.F. 1993. Fumonisin B1, B2, and B3 content of Iowa, Wisconsin, and Illinois corn and corn screenings. *Journal of Agricultural and Food Chemistry*, 41(2):263-266.
81. Mwanza, M. 2012. A comparative study of fungi and mycotoxin contamination in animal products from selected rural and urban areas of South Africa with particular reference to the impact of this on the health of rural black people. University of Johannesburg.
82. Nawaz, S., Scudamore, K.A. & Rainbird, S.C. 1997. Mycotoxins in ingredients of animal feeding stuffs: I. Determination of Alternaria mycotoxins in oilseed rape meal and sunflower seed meal. *Food Additives & Contaminants*, 14(3):249-262.
83. Nelson, P.E., Desjardins, A.E. & Plattner, R.D. 1993. Fumonisins, mycotoxins produced by Fusarium species: biology, chemistry, and significance. *Annual review of phytopathology*, 31(1):233-252.
84. Nelson, P.E., Dignani, M.C. & Anaissie, E.J. 1994. Taxonomy, biology, and clinical aspects of Fusarium species. *Clinical microbiology reviews*, 7(4):479-504.
85. Newton, A.C., Flavell, A.J., George, T.S., Leat, P., Mullholland, B., Ramsay, L., Revoredo-Giha, C., Russell, J., Steffenson, B.J. & Swanston, J.S. 2011. Crops that feed the world 4. Barley: a resilient crop? Strengths and weaknesses in the context of food security. *Food Security*, 3(2):141.
86. Nielsen, K.F. & Thrane, U. 2000. Fast methods for screening of trichothecenes in fungal cultures using GC-MS/MS. *Mycotoxin research*, 16:252-256.
87. Norred, W.P. & Voss, K.A. 1994. Toxicity and role of fumonisins in animal diseases and human esophageal cancer. *Journal of Food Protection*, 57(6):522-527.
88. Pavlović, M., Plestina, R. & Krogh, P. 1979. Ochratoxin A contamination of foodstuffs in an area with Balkan (endemic) nephropathy. *APMIS*, 87(1-6):243-246.
89. Pitt, J. & Hocking, A. 1997. Fungi and Food Spoilage Blackie Academic & Professional. *New South Wales, Australia*.

90. Prandini, A., Tansini, G., Sigolo, S., Filippi, L., Laporta, M. & Piva, G. 2009. On the occurrence of aflatoxin M 1 in milk and dairy products. *Food and Chemical Toxicology*, 47(5):984-991.
91. Prieto-Simón, B., Noguer, T. & Campas, M. 2007. Emerging biotools for assessment of mycotoxins in the past decade. *TrAC Trends in Analytical Chemistry*, 26(7):689-702.
92. Rehman, S. & Mohandes, M. 2008. Artificial neural network estimation of global solar radiation using air temperature and relative humidity. *Energy Policy*, 36(2):571-576.
93. Reiter, E.V., Cichna-Markl, M., Chung, D.H., Zentek, J. & Razzazi-Fazeli, E. 2009. Immuno-ultrafiltration as a new strategy in sample clean-up of aflatoxins. *Journal of separation science*, 32(10):1729-1739.
94. Richard, E., Heutte, N., Bouchart, V. & Garon, D. 2009. Evaluation of fungal contamination and mycotoxin production in maize silage. *Animal feed science and technology*, 148(2):309-320.
95. Richard, J.L. 2007. Some major mycotoxins and their mycotoxicoses—An overview. *International journal of food microbiology*, 119(1):3-10.
96. Richards, C.J. & Hicks, B. 2007. Processing of corn and sorghum for feedlot cattle. *Veterinary Clinics of North America: Food Animal Practice*, 23(2):207-221.
97. Roberts, T., Cordier, J.-L., Gram, L., Tompkin, R., Pitt, J., Gorris, L. & Swanson, K. 2005. Feeds and pet foods. Micro-Organisms in Foods 6. Springer. p. 250-276).
98. Saitou, N. & Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular biology and evolution*, 4(4):406-425.
99. Shadmi, A., Volcani, R. & Nobel, T. 1974. The pathogenic effects on animals fed with mouldy hay or given its etheric fraction. *Transboundary and Emerging Diseases*, 21(7):544-552.
100. Shah, H.U., Simpson, T.J., Alam, S., Khattak, K.F. & Perveen, S. 2010. Mould incidence and mycotoxin contamination in maize kernels from Swat Valley, North West Frontier Province of Pakistan. *Food and Chemical Toxicology*, 48(4):1111-1116.
101. Shephard, G.S. 2009. Aflatoxin analysis at the beginning of the twenty-first century. *Analytical and bioanalytical Chemistry*, 395(5):1215-1224.
102. Sivakumar, V., Singaravelu, G. & Sivamani, P. 2014. Isolation, Characterization and Growth Optimization of Toxicogenic Molds from Different Animal Feeds in Tamilnadu. *Int. J. Curr. Microbiol. App. Sci*, 3(9):430-445.
103. Smalley, E. 1991. Identification of mycotoxin producing fungi and conditions leading to aflatoxin contamination of stored foodgrains.
104. Smith, D. & Henderson, R. 1991. Mycotoxins and animal foods: CRC press.
105. Stoev, S.D. 2010. Studies on some feed additives and materials giving partial protection against the suppressive effect of ochratoxin A on egg production of laying hens. *Research in veterinary science*, 88(3):486-491.
106. Stoev, S.D., Dutton, M.F., Njobeh, P.B., Mosonik, J.S. & Steenkamp, P.A. 2010. Mycotoxic nephropathy in Bulgarian pigs and chickens: complex aetiology and similarity to Balkan Endemic Nephropathy. *Food additives and contaminants*, 27(1):72-88.
107. Sudekum, K., Schroder, A., Fiebelkorn, S., Schwer, R. & Thalmann, A. 2008. Quality characteristics of pelleted compound feeds under varying storage conditions as influenced by purity and concentration of glycerol from biodiesel production. *Journal of Animal and Feed Sciences*, 17(1):120.
108. Sultana, N. & Hanif, N. 2009. Mycotoxin contamination in cattle feed and feed ingredients. *Pakistan Veterinary Journal*, 29(4):211-213.
109. Sweeney, M.J. & Dobson, A.D. 1998. Mycotoxin production by Aspergillus, Fusarium and Penicillium species. *International journal of food microbiology*, 43(3):141-158.
110. Sydenham, E.W., Thiel, P.G., Marasas, W.F., Shephard, G.S., Van Schalkwyk, D.J. & Koch, K.R. 1990. Natural occurrence of some Fusarium mycotoxins in corn from low and high esophageal cancer prevalence areas of the Transkei, Southern Africa. *Journal of Agricultural and Food Chemistry*, 38(10):1900-1903.
111. Tamura, K., Nei, M. & Kumar, S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences of the United States of America*, 101(30):11030-11035.

112. Toscani, T., Moseriti, A., Dossena, A., Dall'Asta, C., Simoncini, N. & Virgili, R. 2007. Determination of ochratoxin A in dry-cured meat products by a HPLC–FLD quantitative method. *Journal of Chromatography B*, 855(2):242-248.
113. Trucksess, M.W., Stack, M.E., Nesheim, S., Page, S.W., Albert, R.H., Hansen, T.J. & Donahue, K.F. 1991. Immunoaffinity column coupled with solution fluorometry or liquid chromatography postcolumn derivatization for determination of aflatoxins in corn, peanuts, and peanut butter: collaborative study. *Journal-Association of Official Analytical Chemists*, 74(1):81-88.
114. Turner, P., Gong, Y., Pourshams, A., Jafari, E., Routledge, M., Malekzadeh, R., Wild, C., Boffetta, P. & Islami, F. 2012. A pilot survey for Fusarium mycotoxin biomarkers in women from Golestan, Northern Iran. *World Mycotoxin Journal*, 5(2):195-199.
115. Tyasi, T.L., Gxasheka, M. & Tlabelac, C.P. 2003. Assessing the effect of nutrition on milk composition of dairy cows: A review.
116. Van der Merwe, K., Steyn, P., Fourie, L., Scott, D.B. & Theron, J. 1965. Ochratoxin A, a toxic metabolite produced by Aspergillus ochraceus Wilh. *Nature*, 205(4976):1112-1113.
117. Verstraete, F. 2008. European Union Legislation on mycotoxins in food and feed. Overview of the decision-making process and recent and future developments. *Mycotoxins: Detection methods, management, public health and agricultural trade*:77-99.
118. Wagacha, J. & Muthomi, J. 2008. Mycotoxin problem in Africa: current status, implications to food safety and health and possible management strategies. *International journal of food microbiology*, 124(1):1-12.
119. Whitaker, T., Dickens, J. & Monroe, R. 1979. Variability associated with testing corn for aflatoxin. *Journal of the American Oil Chemists' Society*, 56(9):789-794.
120. Whitlow, L. & Hagler, W. 2002. Mycotoxins in feeds. *Feedstuffs*, 74(28):1-10.
121. Withanage, G., Murata, H., Koyama, T. & Ishiwata, I. 2001. Agonistic and antagonistic effects of zearalenone, an etrogenic mycotoxin, on SKN, HHUA, and HepG2 human cancer cell lines. *Veterinary and human toxicology*, 43(1):6-10.
122. Zachariasova, M., Dzuman, Z., Veprikova, Z., Hajkova, K., Jiru, M., Vaclavikova, M., Zachariasova, A., Pospichalova, M., Florian, M. & Hajslova, J. 2014. Occurrence of multiple mycotoxins in European feedingstuffs, assessment of dietary intake by farm animals. *Animal feed science and technology*, 193:124-140.
123. Zain, M.E. 2011. Impact of mycotoxins on humans and animals. *Journal of Saudi Chemical Society*, 15(2):129-144.
124. Zaki, M.M., El Madany, S.A., Shaheen, H.M and Rizzi , L. 2012. Mycotoxins in Animal: Occurrence, effects, prevention and management. Journal of Toxicology and Environmental Health Sciences, 4(1): 12-28

LIST OF ANNEXURE

Emerging farm - Annexure: 1

SECTION A. DEMOGRAPHIC CHARACTERISTICS

1. Farmers Details:
   ……

2. Name of area/ Coordinate points:
   ……...

3. Gender	4. Age	5. Marital status	6. Educational level
a) Female b) Male	a) Below 20 b) 20-30 c) 31-40 d) 41-50 e) 51-60 f) above	a) Married b) Single c) Divorced d) Widowed	a) Primary level b) Secondary level c) Tertiary level d) Postgraduate e) No formal education
7. Race	8. Working status	9. Total number of people in household	10. Land ownership
a) Black b) White c) Coloured	a) Employed b) Self-employed c) Unemployed d) Looking for job e) Pensioner	Males ☐ Females ☐	a) Own b) Lease c) Communal

SECTION B. LIVESTOCK INFORMATION

1. Which livestock animals do you keep?

Cattle	Sheep	Goats	Swine	Poultry	Others	All

2. For which purpose do you keep them for?

Income generation	Consumption	Other

3. Do you vaccinate?

Yes	No

4. If YES, who vaccinate your livestock animals?

State veterinarian	Animal Health Technician	Owner	Helper

5. How often do your livestock animals get checked by the veterinarian?

Annually	Monthly	Weekly	Daily	Other

6. Any disease encountered before on the farm?

Yes	No

7. If yes, which one?

FMD	Brucellosis	TB	BVD	LSD	Others

SECTION C. FEEDING INFORMATION

1. Do you supplement your livestock animals?

Yes	No

2. If yes, with what?

Mineral licks	Injectable minerals	Proteins	Others

3. Do you either purchase or self-prepare your supplements?

Purchase	Self-prepared

4. Type of feed do you feed your livestock animals?

Roughages	Concentrates	Mixed feeds

SECTION D. WEATHER AND STORAGE TYPE

1. Which weather condition is mostly experienced this side?

Dry	Humid	Rainy	Others

2. Which type of storage system do you use?

Open storage	Closed storage

3. How do you preserve your feeds?

Bags	Dustbins	Ground	Others

4. Do you know anything about feed contamination?

Yes	No

5. Do you know anything about mycotoxin?

Yes	No

6. If YES, from where did you learn about it?

Radio	TV	Magazines	Newspaper	Others

7. Do you know that mycotoxins are carcinogenic?

Yes	No

8. Would you like to know more about Mycotoxins and their effects?

Yes	No

Thank you very much for your cooperation and participation in this study.

Commercial supplier - Annexure: 2

SECTION A: COMPANY DETAILS

1. Company name: ……...
2. Company address:
   ……..
   ……..
   ……..
   ……..

SECTION B: COMPANY RECORDS

1. How long has the company been operating?
   ……
2. Which type of feed products do you supply?
   ……
3. Whom do you supply your product to?
   ……
4. Do you supply the product produced by you?

   Yes No
5. If YES, which ones?
   ……..
6. Perhaps do you also supply product produced by other?

   Yes No
7. If YES, which ones?
   ……..
8. Does your product adapt to the related South African or other standards and law?

   Yes No
9. If yes, to what applicable standard or law does your product adapt?
   ……
   ……
   ……

SECTION C: INSPECTION INFORMATION

1. How often are your product inspected?
   ……..
2. Which method of inspection does your inspector use?
   ……..

3. Is the floor of the storage inspected daily?

……..

4. Which hygienic method do you use to keep the floor sanitized?

……..

SECTION D: STORAGE INFORMATION

1. What type of storage does the company use?

…….....

2. At what temperature regulation do you keep your product?

……..

3. How long do you keep the product before supplying it?

…….

4. Is the area where you keep your product always dry?

Yes No

5. If YES, what do you do to maintain that?

……
……
…….

6. Which mode of ventilation system do you use?

…….

7. If YES, how often is it inspected?

……..

8. Do rodents enter into your storage area?

Yes No

9. If YES, how do you control them?

……
……
……
……

10. If NO, which measures have you taken to prevent them from entering?

……
……
……
…….

E. PACKAGING INFORMATION.

1. Which type of packaging is best for your produced product?

……

2. Who is supplying you with your packaging material?

……

3. Which shelf method do you use to store your product?

Metal	Wood

4. Do your company do drop-shipping?

Yes	No

5. If YES, how far do you transport your product?

……..

6. Which mode of transport do you use?

Closed	Open

Thank you for your kindly assistance