Incidence of Fungi and Mycotoxins in dairy cattle Feeds from some selected Smallholder Farms in South Africa

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By

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ABSTRACT

Dairy feed is an indispensable part of the dairy industry, essential for high-quality and nutritious milk. These feeds are vulnerable to contamination by a diverse range of mycoflora, that produce several mycotoxins, causing severe feed quality loss and posing a significant challenge to animal and human health. The aim of this present study was to determine the safety levels of 70 dairy cattle feeds and feed ingredients sourced from some selected smallholder dairy farms in the Free State and Limpopo provinces of South Africa during two seasons (summer and winter) from 2018 to 2019 regarding fungal contamination and to evaluate the effects of seasonal and geographical variation on the mycotoxigenicity of the isolated fungal species. The feeds were screened for fungal contamination following both macro- and microscopic methods, and their identities were confirmed by molecular means. Additionally, mycotoxins produced by the isolated mycotoxigenic fungal species were analysed using liquid chromatography with tandem mass spectrometry (LC-MS/MS). In this study, a total of 237 fungal isolates from 14 genera were isolated from the dairy feeds and feed ingredients. Also, mean fungal loads recorded in the feeds ranged from 9.3×10^3 to 3.6×10^5 CFU/g in the Free State and Limpopo provinces, respectively. Multivariate analysis of variance (MANOVA) showed that none of the single factors (season or province) had a significant effect on the mycotoxins production capacity of the isolated fungal species. However, levels of AFB_1 (0.22 to 10445.8 µg/kg) produced during summer was higher than in winter (0.69 to 190.22 µg/kg). The same trend was observed for AFB_2 in the summer (0.11 to 3.44 µg/kg) and winter (0.21 to 2.82 µg/kg). Furthermore, maximum and minimum zearalenone (ZEN) concentrations (97.18 and 5.20 µg/kg) were observed in the Limpopo summer and Free State winter samples, respectively. Lastly, the mycotoxogenic fungal species failed to produce other mycotoxins tested for. Therefore, since milk is majorly consumed in different forms, the high prevalence of mycotoxigenic fungi and mycotoxins recorded in this present work is a matter of concern to the health of the dairy cattle and consumers of dairy milk and milk by-products in South Africa.

Keywords: Dairy feed, milk, fungal loads, mycotoxins, LC-MS/MS.

DECLARATION

I, Oluwasola Abayomi Adelusi, hereby declared that this dissertation is a product of my work, carried out under the supervision of Prof. Patrick B. Njobeh and Dr. Janet A. Adebiyi, and that this work has not been submitted for any academic degree at any other University.

Oluwasola Abayomi Adelusi

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Prof. Patrick Berka Njobeh Dr. Janet Adeyinka Adebiyi

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DEDICATION

To my Lord and saviour, the covenant-keeping God, I thank you so much for the courage, strength, grace, and protection you bestowed upon me throughout my study time. My wife, Deborah Farinloye Adelusi, thank you for your love. In the loving memory of my late beloved father, Mr. Folarin Osho Adelusi. This dissertation is dedicated to my sweet and caring mother, Mrs. Florence Sidikat Adelusi, whose day-and-night prayer, love, support, and words of encouragement help me to this stage of my career. To my siblings, Bukola Owoeye, Funmilayo Adelusi, Bosede Adelusi, Oluwatosin Adelusi, and Opeyemi Adelusi, thank you for believing in me to accomplish my dream. You are the best I ever could ask for; may God bless you all.

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LIST OF ABBREVIATIONS

AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFM_2	Aflatoxin M_2
AFs	Aflatoxins
ALT	Altenuen
AME	Alternariol monomethyl ether
AOH	Alternariol
a_w	Water activity
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
CAST	Council for Agricultural Science and Technology
CFU	Colony forming unit
CIT	Citrinin
CPA	Cyclopiazonic acid
CTA	Technical Centre for Agricultural and Rural Cooperation
CYA	Czapek yeast agar
DAFF	Department of Agricultural, Forestry and Fisheries
DCM	Dichloromethane
DEP	Dichloromethane-ethyl acetate-propan-2-ol
DF	Degree of freedom
DL	Desolvation line
DNA	Deoxyribonucleic Acid
DON	Deoxynivalenol
EC	European Commission
EFSA	European Food Safety Authority

ESI	Electron ionisation
EU	European Union
FAO	Food and Agricultural Organisation
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Funonisin B_3
FBs	Fumonisins
FHB	*Fusarium* head blight
GDP	Gross Domestic Product
HIV	Human immunodeficiency virus
HT-2	HT-2 toxin
IARC	International Agency for Research on Cancer
IBD	Inflammatory bowel disease
IFAD	International Fund for Agricultural Development
ISR	Induced systemic resistance
ITS	Internal transcribed spacer
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MANOVA	Multivariate Analysis of Variance
MEA	Malt extract agar
MMC	Matrix-matched calibration curves
MPA	Mycophenolic acid
MPO	Milk Producers Organisation
MRM	Multiple reaction monitoring
MS	Mean square
NCBI	National Center for Biotechnology Information
OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C

OTs	Ochratoxins
P	Probability
PAT	Patulin
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
R^2	Coefficient of determination
R_F	Retardation factor
RNA	Ribonucleic acid
Spp	Species
SSA	Sub-Saharan Africa
T-2	T-2 Toxin
TB	Tuberculosis
TeA	Tenuazonic acid
TEF	Toluene-ethyl acetate-formic acid
THs	Trichothecenes
TLC	Thin layer chromatography
TMR	Total mixed ration
US	United State
USFDA	United State Food and Drug Administration
UV	Ultraviolet
VR	Variance ratio
WHO	World Health Organisation
ZAR	South African Rand
ZEN	Zearalenone

LIST OF UNITS

%	Percentage
^{o}C	Degrees Celsius
µg/kg	Microgram per kilogram
µg	Microgram
µL	Microlitre
µm	Micromitre
CFU/g	Colony forming units/gram of sample
cm	Centimetre
g	Gram
Kg	Kilogram
L	Litre
l/km^2	Litre/kilometre square
L/min	Litre/minute
M	Molar
mg	Milligram
Min	Minute
mL	Millilitre
mm	Millimetre
ng	Nanogram
sec	Second
V	Volume

CHAPTER ONE
GENERAL INTRODUCTION

1.1 BACKGROUND

Milk and milk products play an essential role in human nutrition due to a wide range of essential nutrients present in them, which are relevant to human and animal health (Kawonga *et al*., 2012). While all mammals can produce milk, cattle accounts for more than 90% of the world's milk production (McGuffey and Sherley, 2011). FAO (2021) reported that the estimated world milk output in 2020 was around 906 million tons, 2.0% higher than the previous year, driven by production growth across geographical regions, except in Africa, where milk production remained stagnant at 49 million tons due to declination registered in some of her major producing countries like Kenya, Morocco, and South Africa. Dairy cattle milk production decreased slightly in South Africa due to dry weather conditions and rising feed prices, which reduced farm profits (FAO, 2021).

The dairy industry is essential to the South African labour market, being the country's fifth-largest agricultural industry and contributing approximately 14.5 billion ZAR (South African Rand) each year to the nation's Gross Domestic Product (GDP). This agricultural section has about 1,680 milk producers employing 45,000 workers and providing support to over 120,000 individuals across the country (DAFF, 2012a; MPO, 2017). South Africa's cattle milk production comes from both commercial and smallholder farms. The main differences between these dairy sub-sectors are the genotypes of cattle raised, farm size, number of cattle, and management level. Smallholder dairy production is a relatively low agricultural system in which farmers engage in production at a less developed and capital-intensive level in contrasts to established commercial farmers. This dairy sub-section includes both communal and emerging farmers. Traub (2015) defined communal farmers as those who practice subsistence farming on communally owned farmlands allocated to them by traditional leaders, with lesser dairy cows and subsequent low yield, while emerging farmers according to Muntswu *et al*. (2017), are those who benefit from land reform programmes with more than 15 milking cattle grazing on 1 hectare of land and producing at least 100 litres of milk daily.

However, one of the most significant impediments to smallholder dairy productivity in sub-Saharan African countries (SSA) is shortage of good quality feeds. In addition to forages and cereals, dairy cattle farmers purchase other feeds like oilseeds, industrial by-products such as brewer's dry grain, molasses, bone meal, among others, to complement their locally made feeds (Ojango *et al*., 2017). Proper feed management is required for good health and welfare of lactating cows. Unfortunately, most dairy cattle farmers in SSA lack access to high-quality animal feeds and good farm management measures, as well as animal nutrition. (VanLeeuwen *et al*., 2012; Nyka *et al*., 2014). Indeed, dairy management practices in this region centred majorly on increasing production and yield with little or no concern for the safety of milk and dairy products, which are compromised when animals are allowed to feed on contaminated feeds.

Among feed and feed ingredient contaminants, contamination with fungi capable of producing mycotoxins is one of the main challenges to livestock production, including dairy cattle in South Africa, due to the mycotoxins they may produce in these feed materials. Mycotoxins are harmful substances produced by some organisms in the kingdom fungi that contaminate agricultural commodities, resulting in detrimental effects on animal and human health, as well as animal productivity. Mycotoxin production occurs under favourable conditions that allow fungi to grow on feeds and feedstuffs in the field during harvest, storage as well as feed processing and transit (Mwende *et al*., 2016).

Fusarium, Aspergillus, Alternaria, Penicillium, and *Claviceps* are the most common fungal genera capable of producing mycotoxins. These mycotoxin producing fungi are divided into two groups viz: field fungi (which invade plants and produce mycotoxins mainly on the field), for example, *Fusarium* and *Alternaria* spp., and storage fungi (which colonise agricultural commodities and produce toxins during storage), such as *Penicillium* and *Aspergillus* spp. (Kemboi *et al*., 2020). Among fungal genera, *Penicillium*, *Aspergillus*, and *Fusarium* are generally known as the most challenging contaminants of foods and feeds (Alhannaq and Yu, 2017), with them being the most prevalent feed contaminants in South Africa (Iheanacho *et al*., 2014). Additionally, mycotoxins such as aflatoxins (AFs) produced by the genera *Aspergillus*, deoxynivalenol (DON), zearalenone (ZEN), HT-2 and T-2 toxins, and fumonisins (FBs) formed by *Fusarium* spp, as well as ochratoxins (OTA and OTB) formed by *Aspergillus* and *Penicillium* spp, are prevalent mycotoxins

documented regularly in feeds and feedstuffs from South Africa (Changwa *et al*., 2018; 2021; Gruber-Dorninger, *et al*., 2019).

When lactating cows are fed mycotoxin contaminated feed, particularly aflatoxin B_1 (AFB_1), the mycotoxin bio-transforms into aflatoxin M_1 (AFM_1), which accumulates in the animal tissues and contaminate milk and by-products of such bovine (Goncalve *et al*., 2015). Hence, the presence of mycotoxin residues in milk is considered a global threat due to its resilience to high temperatures and physical or chemical treatments. For this reason, feed or food processing operations are insufficient and ineffective for mycotoxin elimination (Pereira *et al*., 2019), resulting in human exposure to these deadly toxicants (Alhannaq and Yu, 2017).

It must be emphasised that fungal diseases are diverse and widespread in South Africa. Unfortunately, about 3.2 million people (7.1%) of the country's 56.5 population are inflicted each year by fungal related infections, triggered mainly by certain diseases like tuberculosis (TB) and Human Immunodeficiency Virus (HIV), as well as poverty syndromes across the nation (Schwartz *et al*., 2019). Also, significant mycotoxins, such as AFs, OTA, and FBs, are not adequately controlled in South African animal diets (Mngadi, *et al*., 2008; Njobeh *et al*., 2012), leading to mycotoxicoses in some animals, including dairy cattle (Botha *et al*., 2014) and dogs (Dutton *et al*., 2012). As a result, it is important to evaluate the incidence and contamination levels of toxigenic fungi, and their attendant toxins in South African dairy cattle feeds regularly.

Even though a few numbers of researches on mycotoxin contamination in dairy cattle feeds and feedstuffs have been carried in South Africa (Mngadi *et al*., 2008; Changwa *et al*., 2018; 2021), little is known about the mycobiota responsible for the production of these toxic compounds. To the best of our knowledge, this work provides the first report on the effects of different geographical locations and seasons on the mycotoxigenicity of fungal spp. recovered from South African smallholder dairy cattle feeds. Therefore, it is the aim of this present study to evaluate and assess the toxigenic potentials of fungi spp. contaminating dairy feeds and feedstuffs in the Free State and Limpopo provinces of South Africa at different seasons.

1.2 PROBLEM STATEMENT

Despite increased awareness of food security as the bedrock for active and healthy living, food insecurity persists, mainly in SSA nations, where a large proportion of the population lacks access to nutritious, affordable, and safe food. It is worth noting that the primary factor threatening food security in this region is postharvest losses. FAO (2011) reported that approximately one-third of all agricultural products is lost yearly. Mycotoxins, produced by toxigenic fungal genera including *Aspergillus, Penicillium,* as well as *Fusarium,* are the main cause of these losses, with *Aspergillus* spp. being the most prevalent contaminants of cattle feeds in this continent (Okun *et al*., 2015; Omeiza *et al*., 2018).

While more than 400 mycotoxins have been reported globally, the South African government only regulates a few in dairy cattle feeds (Kemboi *et al*., 2020). The occurrence of these toxins in dairy cattle feeds poses a twofold risk to dairy cattle. Firstly, they may cause serious harmful effects on dairy cattle health, including nephrotoxicity, hepatotoxicity, immunosuppression, reduced milk yield, decreased feeding efficiency, and low fertility (Khatoon, 2012; Gonçalves *et al*., 2015). Additional effects include abortion, weight loss, laminitis, and impaired rumen function. Secondly, they may jeopardise the food supply chain due to carrying over of mycotoxins from feed to milk (Gizachew *et al*., 2016; Claudious, 2019), thereby impairing the quality of milk and dairy products for human consumption.

Consumption of mycotoxin contaminated milk and other dairy products causes serious human health-related problems since mycotoxins are known to be carcinogenic, immunotoxic, genotoxic, nephrotoxic, and cytotoxic (Janik *et al*., 2020) and may impair immune responses, increasing the risk of secondary infections. In more severe cases, such as prolonged chronic toxicity or high acute intoxication, it may result in death (Omotayo *et al*., 2019). Besides their adverse effects on feed quality and human and animal health, mycotoxins equally cause severe economic losses due to the cost directed towards food safety programmes and are responsible for some barriers to international trade (Enyiukwu *et al*., 2014; Gbashi *et al*., 2018).

Thus, there is a growing need in South Africa for better food and feed management, as well as adequate food and feed testing services, to help monitor the presence and nature of mycotoxin contamination in South African livestock feeds and feed ingredients, especially when some of the mycotoxins have been detected in by-products of animals that consumed mycotoxin contaminated feeds (Dutton *et al.*, 2012; Shephard *et al.*, 2013). Despite the body of information showing South African agricultural products are contaminated regularly with fungi and mycotoxins, studies are still required on dairy cattle feeds. Therefore, it is essential to provide data on mycoflora and mycotoxins of interest in relation to health and productivity of dairy cattle in the country. The data is believed to contribute to the protection of dairy cattle and human health by addressing the mycotoxin contamination problem in animal nutrition, and consequently, in human diets. Additionally, it will create awareness among dairy cattle farmers, feed producers, milk and milk product consumers, and the entire country on the danger of fungi with respect to mycotoxins and associated health and economic impacts.

1.3 HYPOTHESIS

It is hypothesised that smallholder dairy cattle feeds in South Africa may be contaminated by mycotoxin producing fungi. Thus, frequent exposure of dairy cattle to mycotoxins produced by these fungi via contaminated feeds will severely impact animal and human health. It is also assumed that smallholder dairy farmers in the country have a poor understanding of fungal and mycotoxin contamination and the associated health consequences

1.4 RESEARCH QUESTIONS

To assess fungi and mycotoxin exposure in dairy cattle, the following research questions will be addressed:

- What are the fungal species contaminating smallholder dairy cattle feeds and feed ingredients in South Africa?
- What is the level and frequency of fungal contamination in dairy cattle feeds and feedstuffs in the country?

- What are the attendant toxins produced by the toxigenic fungi present in these dairy? cattle feeds, and to what level of contamination?

- What are the effects of different geographical regions and seasonal variation on the mycotoxin production capacity? and
- What possible suggestions based on the responses from the research questions can be made to improve or maintain dairy cattle feeds and feedstuffs quality?

1.5 AIMS AND OBJECTIVES

1.5.1 Aim of the study

This present study aims to evaluate the safety levels of various dairy cattle feeds and feedstuffs in some selected smallholder dairy cattle farms in South Africa with respect to fungi and their toxigenic potentials under different seasons.

1.5.2 Objectives of the study

- To screen for fungi contaminating dairy cattle feeds and feed ingredients from Free State and Limpopo provinces of South Africa.

- To evaluate the incidence of fungi spp. isolated from the dairy cattle feeds and feedstuffs.

- To determine the toxigenicity of fungal spp. recovered from dairy cattle feeds using Liquid chromatography tandem mass spectrometry (LC-MS/MS).

- To assess the effects of seasonal and geographical variation, as well as their interaction on mycotoxin production capacity of the toxigenic fungal species.

CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

Fungi are among the world most prolific organisms, with about 1.5 million species, but only a few (around 70,000) have been described (Blackwell, 2011). These microbes are responsible for over 25% of global food deterioration (Pandya and Arade, 2016). They occur at all trophic levels but are highly abundant in soil, water, and air. As such, they have profound global impacts on ecosystems, agriculture, economies, as well as human and animal health. Mycotoxins are harmful secondary metabolites produced naturally by some organisms in the kingdom fungi that contaminate crops from planting to transit and storage. They are regarded as major contributors to massive agricultural products losses in underdeveloped countries, particularly in sub-Saharan Africa (SSA) (Udomkun *et al*., (2017). Most mycoflora involved in mycotoxin production are mainly from the genera *Aspergillus*, *Fusarium* and *Penicillium*, which regularly contaminate and compromise food safety and quality. Aflatoxins (AFs), trichothecenes (TH), deoxynivalenol (DON), zearalenone (ZEN), ochratoxins (OTs), and fumonisins (FBs) are the prominent fungal toxins (mycotoxins) due to their economic and health significance (Bryden, 2012; Janik *et al*., 2020). Animals and humans are exposed to these toxicants mainly via intake of infected feeds and foods (Alonso, 2013; Goncalves *et al*., 2015), but there are other exposure routes (dermal, parental and aerosol) leading to a variety of health risks (Zain, 2011; da Rocha *et al*., 2014; Sarma *et al*., 2017). This review focused on the common toxigenic fungi, and mycotoxins contaminating dairy cattle feeds and feedstuffs in South Africa, factors influencing their growth and development, the economic impacts of these contaminants on dairy cattle, as well as measures currently adopted to prevent and limit contamination by filamentous fungi and mycotoxins.

2.2 OVERVIEW OF SMALLHOLDER DAIRY CATTLE FARMING IN SOUTH AFRICA

South Africa is located in the southernmost point of Africa, with 122.3 million hectares and about 56.6 million people of diverse ethnic groups and cultures. The country comprises of nine

provinces, i.e., Western Cape, Northwest, Gauteng, Free State, and Northern Cape. Others include Mpumalanga, Limpopo, KwaZulu Natal, and Eastern Cape. It is a country with a wide diversity and abundant rainfall, with some areas experiencing severe drought and extreme heat. The varying climatic conditions in the country, coupled with its topography, favour the growth of a wide range of plants, mainly cereals and feed raw materials such as lucerne, teff, and alfalfa, which are essential feeds and feed ingredients for milk-producing cattle. Even though milk is produced throughout South Africa, dairy farming thrives in the coastal areas due to their abundant rainfall and warm weather, which promote pasture growth and animal production. (DAFF, 2017). Figure 2.1 shows the milk production density among the nine provinces in South Africa.

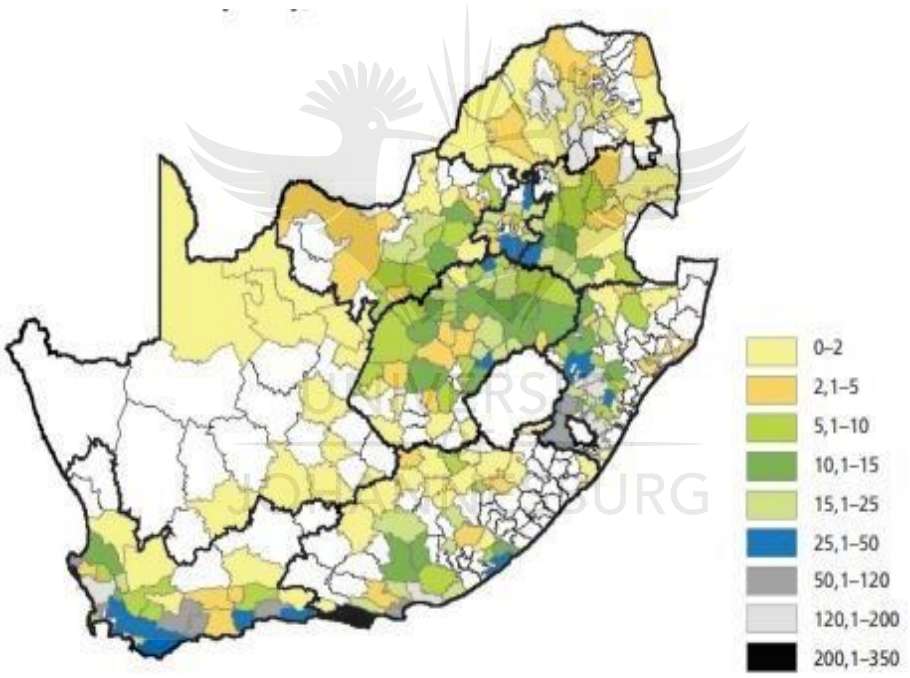


Figure 2.1: Milk production density (l/km) in South Africa (Source: MPO, 2016)

The dairy industry is essential to the South African labour market, with over 1,600 milk producers and 45,000 workers. It is the country's fifth-largest agricultural industry, contributing around

R14.5 billion annually to the nation's GDP and providing financial support for around 120,000 people (DAFF, 2012a; MPO, 2017). Milk production in South Africa is from both commercial and smallholder farms. The main differences between these dairy sub-sectors are the genotypes of cattle raised, farm size, number of cattle, and management levels. According to Baloyi (2010), smallholder dairy farms are less established and poorly resourceful with fewer dairy cattle compared to commercial dairy farms, which are capital intensive and well developed with large sizes of herds. Smallholder dairy farming is categorised into two, namely, emerging and communal farmers. Emerging farmers, as described by Muntswu *et al*. (2017), are those who benefited from land reform programmes, possessing over 15 milking cattle on one hectare of farmland and with at least 100 litres of milk produced each day, while Pienaar and Traub (2015) defined communal farmers as those who practice farming for subsistence on communally owned farmlands obtained from traditional leaders, with smaller cattle sizes and low output.

There are approximately 500 million smallholder farms available worldwide (IFAD, 2002), with a number of them situated in South Africa (Mapekula *et al*., 2011), most of which are in the Eastern and Northern Free state, the Eastern and Western Cape, the Kwazulu-Natal midlands, Gauteng, and Southern parts of Mpumalanga (Department of Agriculture, Forestry and Fisheries 2011; 2014). Furthermore, smallholder farmers own more than 40% of the 1.4 million dairy cattle available in the country (Meissner, 2013). The most popular dairy breeds in South Africa, as reported by Lassen (2012) are Ayrshires, Jersey, Guernsey, and Holstein, however, as established by Mapekula *et al*. (2011), some smallholder farmers keep crossbreds between indigenous and foreign breeds, which may not have been bred for milk production, thus reducing milk productivity. The South African government has long recognised this agricultural sub-sector as a means of achieving poverty reduction and rural development goals and has, therefore, implemented several projects and programmes directed towards this end (Pienaar and Traub, 2015). Dairy cattle performed admirably in milk yield, with adequate quality feed supply and good hygienic conditions (Mellado *et al*., 2011), but dairy cattle in Free State and Limpopo provinces are typically housed in unsanitary environments (Figure 2.2).

Figure 2.2: A typical dairy cattle pen under unhygienic conditions in Free State (*top*) and Limpopo (*bottom*).

2.3 DAIRY CATTLE FEEDS: DEFINITIONS AND CONCEPTS

Feed is any substance, either processed or unprocessed, consumed by animals to meet their nutritional needs (EC, 2011), while feedstuff refers to the raw material or ingredient (of animal or vegetable origin) that is natural, fresh, or processed, used in the formulation of compound feed intended for animal consumption. Generally, the composition of basic components in animal diets varies depending on the animal's age, sex, species, and purpose of rearing (Figen and Zümrüt, 2018). Dairy cattle feeds are supplied to dairy cattle to provide the necessary nutrients (energy, amino acids, minerals, vitamins, and other nutrients) needed for dairy production. However, nutrient demand for dairy cattle differs depending on the gestation and lactation stage (Goncalves *et al*., 2015). In general, dairy cattle feeds are classified into two based on their composition, i.e., roughages and concentrates. Roughages are bulky feeds with low nutritional value, but high crude fibre content (over 18% dry matter) required to stimulate ruminal digestion (Weiss *et al*., 2017), with examples including fresh, dried, or ensiled forages from maize stalk, grasses, and lucerne to by-product feeds.

In contrast, concentrates are high energy, low fibre (less than 18%of dry matter), and high palatable feeds. Concentrates may be high in protein, referred to as protein concentrates such as meat and meat, bone meal, oilseed cakes, feather meal, and fish meal, or high in energy, referred to as energy concentrates including cereals (corn, sorghum, barley, and wheat) and milling by-products. It has been noted that concentrates have higher nutritional contents than an equivalent amount of natural fodder (Lima *et al*., 2011) because they ferment faster in the rumen than forages, making them vital feed ingredients for formulating diets that enhance milk production. Roughages of various types can be blended with concentrate components to generate a total mixed ration (TMR) or a complete ratio to satisfy the nutritional needs of animals.

It is worth noting that feeds are essential, not just to the feed manufacturers and animal producers, but also to the policymakers, regulators, processors, and the consumers of the end-products. Feed management is thus needed for the good health and welfare of dairy cattle because dairy feed is one of the most relevant links in the food supply chain and is key to the economic and efficient production of high-quality food. The quality and type of diets fed to dairy animals by the farmers,

according to Erickson *et al*. (2020), can help boost their annual milk production and income. Gabriel and Puleng (2013) further reviewed that a good dietary strategy is an excellent measure to counteract the effects of fungi and mycotoxin in animals. It is therefore important to maintain the good quality of feeds as well as the nutrients embedded in them by harvesting them at the appropriate time and storing them properly, as keeping them too long on the farm sites, mishandling, and improper storage of the feeds can lead to their deterioration by field and storage fungi (Alonso *et al*., 2013), which attack crops in the field and during storage. Unfortunately, dairy cattle farmers in Free State and Limpopo provinces leave their maize too long on the farm sites before making silage from them, while other feeds and feedstuffs are not properly stored (Figure 2.3), exposing them to fungal and mycotoxins contamination.

Figure 2.3: Illustration of poor storage conditions of hays in the Free State province (*top*) and maize kept too long on the farm site in Limpopo province (*bottom*) with the possibility of fungal and mycotoxin contamination.

2.4 FUNGI

Fungi are highly diversified eukaryotic organisms possessing animal and plant features but classified as a separate kingdom (Phoku 2014). Before the advancement of DNA technology, fungi were believed to be an offshoot of the plant kingdom, however, DNA and biochemical studies have established that fungi are a distinct group of eukaryotes, characterised by their characteristic glucan and chitin cell walls, which frequently surround multinucleated cells. Fungi can be unicellular, like yeasts, or form a network of filaments called mycelium, generally referred to as moulds. Fungal species reproduce by asexual and sexual reproduction cycles and exhibit an alternation of generations. These microbes depend on other organisms for survival by invading and exhibiting them (Njobeh, 2009). Thus, they cannot digest their food, as opposed to other organisms. Nonetheless, they feed by absorbing nutrients from their surroundings, achieved by proliferating through and within the substrates they colonised (Phoku, 2014). Fungi have critical roles in all terrestrial ecosystems as decomposers, food sources and opportunistic pathogens (Wood, 2017).

The number of fungal species is unknown due to less information available to them than the relatively well-known plant and animal kingdoms. The number of fungi originally predicted to exist in nature were 1.5 million species (Hawksworth, 2001), but high-throughput sequencing methods allowed for a more precise estimate of 5.1 million species (Blackwell, 2011). The fungal population in South Africa is unknown, although the plant to fungi ratio generated based on international research suggests that there could be at least 171,500 indigenous species (Wood, 2017). Also, information on fungal introduction routes into the country is limited. It was presumed that most fungal species were introduced to South Africa as passengers with crop plants (Wood, 2017).

The global colonisation of food and feed by fungi and their spores during pre-and post- harvest has consistently been reported (Claudious, 2019; Bouti *et al*., 2020; Esan *et al*., 2020). These organisms reduced feed quality, mainly forages and concentrates, by lowering their dry matter content, causing sour flavours and caking. Inhalation or ingestion of spores from mouldy feeds and feedstuffs can cause severe illnesses generally termed "mycosis". Examples of mycoses in

dairy cattle include mycotic abortion and ringworm. The latter may occur in cattle due to the systemic transmission and subsequent proliferation in placental and foetal tissues. Humans may be exposed to these mycoflora when they consume fungal contaminated food or exposed to air and dust containing fungal spores.

Some fungi species produce different beneficial secondary compounds, including, organic acids, antibiotics, alcohols, enzymes, amino acids, growth-promoting compounds, biological pesticides, and those deemed harmful to humans and animals. A group, collectively known as mycotoxins (Ráduly *et al*., 2020), are difficult to eliminate even during food and feed processing. Fungal proliferation and mycotoxin production may occur under favourable temperature, oxygen availability, moisture, insect invasion, relative humidity, and mechanically damage to the host (Hassan *et al*., 2020). Mycotoxins are produced by *Fusarium, Penicillium, Aspergillus, Alternaria, Claviceps,* and *Stachybotrys,* among which *Penicillium, Aspergillus,* and *Fusarium* are the most common pathogens of feeds and foods in South Africa (Ndlovu and Dutton, 2013; Adekoya *et al*., 2018; Greeff-Laubscher *et al*., 2018). Southern African agricultural zones include subtropical or tropical climates, characterised by humid weather, unpredictable rains, and frequent droughts, ideal for fungal development (Darwish *et al*., 2014). Major toxigenic fungi contaminating foods and feeds in South Africa will be discussed subsequently.

2.4.1 Aspergillus

Aspergillus species are the most abundant and pervasive fungi on the planet, comprising 6 subgenera, multiple sections and over 400 known species, 20 of which are human pathogens (Blackwell *et al*., 2005). In South Africa, the genus *Aspergillus* is relatively diverse, consisting of 63 identified species that belong to 11 sections, among which 7 were reported to be new and have been described (Visagie and Houbraken, 2020). *Aspergillus* species reproduces by producing mitotic spores (conidiospores) (Figure 2.4), which contain huge, thick-walled stipes with fruiting bodies known as vesicles (Klich, 2009). The genus is regarded as storage fungi due to their infrequent infection of pre-harvest crops. They are found everywhere (Spadea and Giannico, 2018), particularly in tropical or subtropical regions (Cheli *et al*., 2013). Among the genus *Aspergillus, A. niger, A. parasiticus, A. fumigatus,* and *A. flavus* are the most prevalent. Others

include *A. nidulan, A. ochracues, A. ustus, A. terreus, A. oryzae, A. melleus,* and *A. tamarii*. Additionally, *A. flavus* and *A. parasiticus* remain the most studied *Aspergillus* spp. globally (Yogendrarajah *el al*., 2015), including South Africa (Passone *et al*., 2012; Iheanacho *et al*., 2014). This could be due to their regular incidence in crops and ability to produce aflatoxins, the most notorious group of mycotoxins and infect humans and animals.

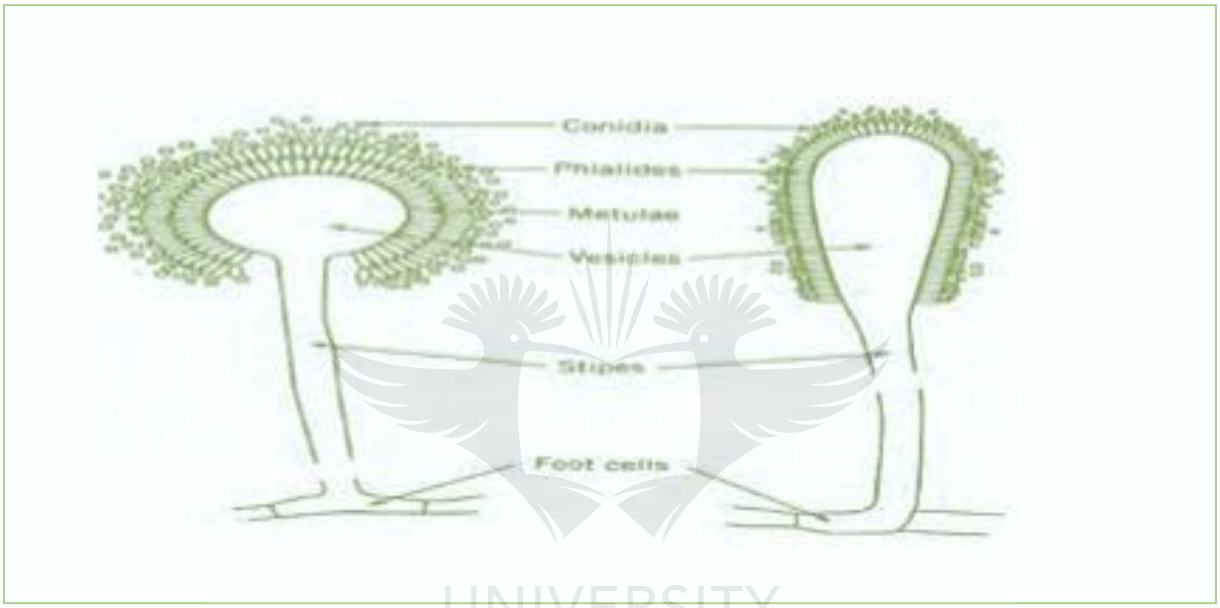


Figure 2.4: Microscopic structure of a typical *Aspergillus* species (Adapted from Klich, 2002).

The genus *Aspergillus* is responsible for producing three of the five agricultural-significant mycotoxins like OTs (OTA, OTB and OTC) (Arroyo-Manzanares *et al*., 2017), FBs (FB_2, FB_4 and FB_6) (Onami *et al*., 2018) and AFs (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1 and AFM_2) (Bennett and Cahill, 2016). OTs are mostly produced by *Aspergillus* spp. from the *Nigri* and *Circumdati* sections (Frisvad *et al*., 2011), additionally, certain species such as *A. niger* from the *Nigri* section are known to produce certain FBs (Frisvad and Larsen 2015a). Moreso, *A. parasiticus* and *A. flavus* are the primary aflatoxigenic species linked with AFs production. However, some other *Aspergilli*

such as *A. nomius* (Yunes *et al*., 2020) and *A. minisclerotigenes* (Adekoya *et al*., 2019) have recently been identified as producers of these lethal toxins. As revealed by Frisvad *et al*. (2019), other economically essential mycotoxins produced by *Aspergillus* spp. include kojic acid, cyclopiazonic acid, speradinen A, versicolorins and tenuazonic acid.

Several *Aspergillus* spp. have been recovered from agricultural products stored under improper storage conditions (Falade, 2011), with temperature of between 19 and 35 ^{o}C (Parra and Magan, 2004) and a_w of 0.8 and 0.9 (Flannigan and Miller, 2001). These are ambient conditions favouring their growth in crops, especially in hot and humid climate zones such as SSA. Relatively high incidence of *Aspergillus* spp. in dairy cattle feeds have been reported in some SSA countries like Kenya (Mwende *et al*., 2016), Ethiopia (Mona *et al*., 2016), Zimbabwe (Claudiou *et al*., 2019; Nleya *et al*., 2021), and in milk products from Egypt (Abdou *et al*., 2017). These group of fungi are diverse but understudied in South Africa (Visagie and Houbraken, 2020). A study by Ndlovu and Dutton (2013) revealed 15 different *Aspergillus* spp. in 82 maize silages, and 21 chopped maize samples (common dairy cattle feeds), the predominant among the *Aspergillus* spp. were *A. fumigatus* (32%), *A. flavus* (21%%) and *A. parasiticus* (20%). Iheanacho *et al*. (2014) also revealed the prevalence of *Aspergillus* isolates in South African compound feeds, with the overall data obtained revealing that 51.1 and 67.5% of feed samples were contaminated with *A. parasiticus* and *A. flavus*, respectively, at high contamination mean level in dairy cattle feeds (4.0×10^4 CFU/g).

Infections caused by *Aspergillus* spp. are among the most frequent filamentous fungal infections (Schwarth *et al*., 2019). Several isolates belonging to the genus *Aspergillus* have been identified as pathogens causing severe aspergillosis in humans and animals (Frisvad and Larsen, 2015b; Visagie and Houbraken, 2020). In South Africa, around 3,885 cases of invasive aspergillosis are recorded each year, owing primarily to the syndemics of tuberculosis, HIV, and poverty (Schwarth *et al*., 2019). Apart from their detrimental effects on animals and humans, *Aspergillus* spp. cause significant farm losses by contaminating food and feed. According to Gbashi *et al*. (2018), the annual cost of *Aspergillus* toxin contamination of African crops is around 750 million USD.

2.4.2 Fusarium

The genus *Fusarium*, discovered over 200 years ago, is a well-known filamentous fungal genus globally. They are member of the phylum Ascomycota, with several morphologically and phylogenetically diverse species found in tropical and subtropical regions (Blackwell et al., 2005). *Fusarium* is characterised morphologically by the presence of fusiform, septate, and semi-circular macroconidia, as well as either or both microconidia of long, multicellular, banana-shaped, or canoe-shaped macroconidia (Phoku, 2014). Many species, however, produce microscopic, single-celled microconidia that range in shape from oval to spherical to fusiform. Their conidia (Figure 2.5) are often airborne or water-borne, with their chlamydospores usually soil-borne (Smith, 2007). Fungi in the genera *Fusarium* are commonly referred to as field or soil fungi because they proliferate during plant growth (Karlsson *et al.*, 2021).

Fusarium species of health and economic importance include *F. culmorum, F. graminearum, F. verticillioides, F. equiseti, F. sporotrichioides, F. proliferatum*, *F. oxysporum,* and *F. avenaceum*. (Mielniczuk and Skwaryło-Bednarz, 2020). Among the genera *Fusarium*, F. *verticillioides* is the most prevalent, associated with crops, particularly maize (Schoeman *et al*., 2018). In addition to their plant pathogenicity, some mycotoxigenic *Fusarium* spp. can produce one or more mycotoxins with different degrees of toxicity (Bountigny *et al*., 2012). For instance, *F. proliferatum* and *F. verticillioides* are the chief producers of FBs, whereas *F. culmorum* and *F.* graminearum are prominent producers of ZEN and TH (Boutigny *et al*., 2012; Phoku, 2014).

Feeds and foods contamination by *Fusarium* spp. have been documented in SSA countries. Egbuta *et al*. (2015) reported a high incidence of *Fusarium* spp. in stored maize from Nigeria with high occurences of *F. proliferatum* and *F. verticillioides*. *F. culmorum* was the first *Fusarium* isolate recovered from South African crops in the 1930s (Beukes *et al*., 2017). Currently, there are about 33 mycotoxigenic *Fusarium* spp associated with crops in the country, notable among these include *F. proliferatum*, *F. subglutinans* and *F. verticillioides* (Beukes *et al*., 2017). Ekwomadu e*t al*. (2018) found *Fusarium* (82%) predominating over other genera like *Penicillium* (63%) and *Aspergillus* (33%) in maize collected from the Northern part of South Africa. In another study conducted in South Africa by Chilaka *et al*. (2012), *Fusarium* spp. were the predominant fungi

isolated from commercial maize. The study showed the predominance of *F. proliferatum* and F. *verticillioides* with incidence rates of 88 and 73%, respectively.

Fusarium head blight (FHB), vascular wilts, seedling blights, cankers, and rot are some of the effects of *Fusarium* on crops (Logrieco *et al*., 2003; Chilaka *et al*., 2017), among these, FHB is the most challenging to crop producers because it reduces cereal yield and quality, and negatively impact food safety (Zeidan *et al*., 2018; Nogueira e*t al*., 2018). Dietary exposure to *Fusarium* toxins, especially FB_1, can lead to chronic bronchitis and inflammatory bowel disease (IBD) in humans (Dorribo *et al*., 2015).

Figure 2.5: Microscopic view of conidia produced by *Fusarium* species (Adapted from Smith, 2007).

2.4.3 Penicillium

Penicillium is one of the largest and most important filamentous fungi, with over 400 recognised species (Visagie *et al*., 2014). Pitt and Hockings (1997) confirmed that *Penicillium* is larger than *Aspergillus* based on number of species. They are ubiquitous and opportunistic saprophytes found almost everywhere. *Penicillium* spp. produce paintbrush-like heads and stalk called conidiophores (Figure 2.6), ending with a branch like a cluster of spores producing cells termed phialides. The blue and green pigments of these spores give the colonies unique colours on food and feed (Pitt and Hocking, 1997). The genus *Penicillium* according to Samson *et al*. (2004), is very difficult to differentiate from each other, although some of its species showed a great deal of intraspecific variability. The most significant foodborne *Penicillium* spp. found in agricultural commodities are *P. oxalicum, P. janthinellum, P. echinulatum, P. chrysogenum, P. marneffei, P. citrinum, P. purpurogenum,* and *P. expansum* (Frisvad and Thrane, 2000).

Penicillium spp in dairy cattle feeds have been documented in South Africa. In 82 maize silages, *Penicillium* spp. was recovered at percentage incidence of 19%, with *P. citricum* and *P. expansum*, the most dominant (Ndlovu and Dutton, 2013). The occurrence of *Penicillium* spp. in maize samples were also reported in South Africa by Ekwomadu *et al*. (2018) at percentage incidence of 63%, with the prevalence of *P. digitatum, P. chrysogenum, P. decumbens*, among others. *Penicillium* spp. can produce several secondary metabolites, including antibiotic, penicillin, or antifungal drug griseofulvin that are useful to humans, and several others which are toxic to humans and animals, called mycotoxins. Mycotoxins produced by *Penicillium* spp. are patulin (PAT), cyclopiazonic acid (CPA), mycophenolic acid (MPA), ochratoxin A (OTA), and citrinin (CIT). Among these toxins, CIT and OTA are the most dangerous to humans and animals, causing acute lesions that can lead to cancer.

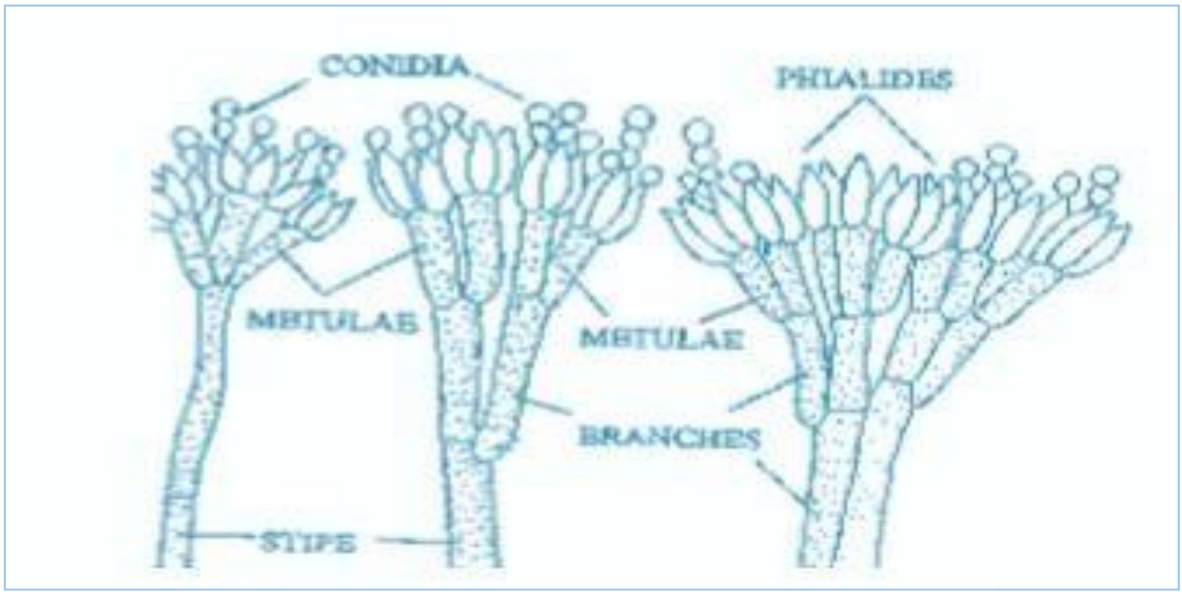


Figure 2.6: Microscopic structures in *Penicillium*, with various forms of branching conidiophore (Adapted from Phoku, 2014).

2.4.4 Alternaria

Alternaria is a large filamentous fungus of the Ascomycota phylum. Nees first described this fungal genus in 1816 (Lawrence *et al*., 2016). *Alternaria* is a field fungus found in humid and semi-dry regions of the world, with species responsible for 20% of agricultural spoilage and 80% of crop losses (Nowicki *et al*., 2012). They are classified as saprotrophs, which means they are primarily involved in the decomposition of organic wastes, or as opportunistic pathogens, causing a variety of animal and human diseases (Barkai-Golan, 2008; Ali *et al*., 2020). The optimal development temperature for this genus spans from 22 to 30 ^{o}C, while the minimum temperature ranges from 2.5 to 6.5 ^{o}C and even lower. *Alternaria* spp. are characterised by the formation of beaked multi-celled coloured spores that are always formed in a dark branching chain (Figure 2.7), with cells longitudinally and transversely divided, giving them a characteristic identification appearance. Although there are approximately 300 species in the genus *Alternaria*, the most

common in various plant commodities are *A. alternata, A. tenuissima, A. radicina, A. brassicae, A. brassicicola, A. arborescent*, and *A. infectoria* (Logrieco *et al*., 2009).

The genus *Alternaria* can colonise several crops, including small-grain cereals, fruits, and vegetables, especially in the phyllosphere (Lee *et al*., 2015). They have been found naturally occurring in various agricultural commodities worldwide (Patriarca *et al*., 2007). Similarly, Ekwomadu *et al*. (2018) isolated *Alternaria* species from South African commercial and small-scale maize, with incidence rates of 30 and 32%, respectively. Furthermore, *Alternari*a spp. have been employed in biological pest control, and it has been shown in a number of studies that the genus *Alternaria* play a significant role in plant induced systemic resistance (ISR) and produce active materials against pests and pathogens (Kaur *et al*., 2019; Fatima *et al*., 2020). Members of these species are also known to produce some poisonous secondary metabolites that cause food and feed poisonings, such as altenuen (ALT), tenuazonic acid (TeA), alternariol (AOH) and monomethyl ether (AME) (EFSA, 2011). Toxins produced by members of this fungi genus can cause reproductive disorder in humans by disrupting the secretion of reproductive hormones, especially steroids and progestin. This was recently reviewed by Anqi *et al*. (2021).

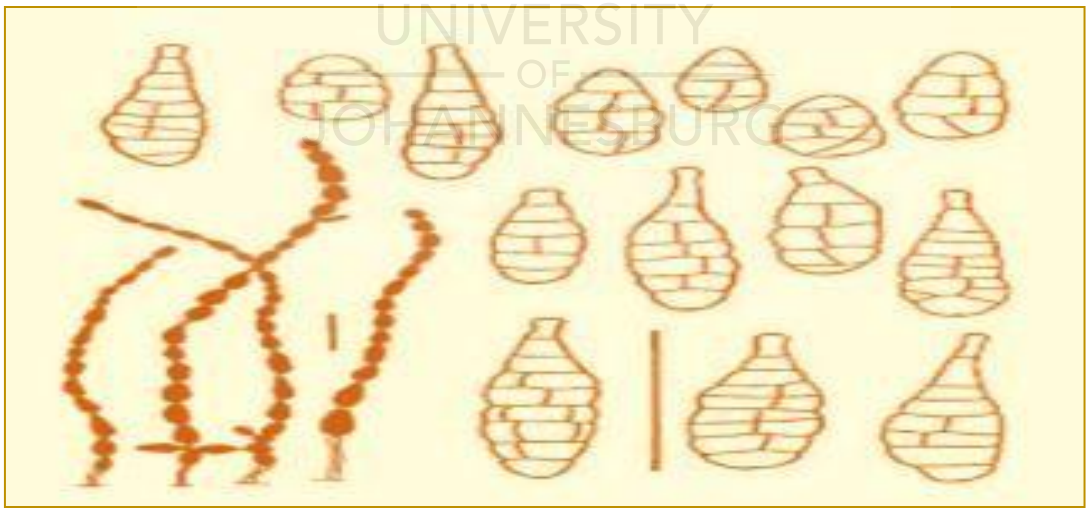


Figure 2.7 Microscopic structures of *Alternaria* spores (Adapted from Taralova *et al*., 2011).

2.5 MYCOTOXINS

Mycotoxins are harmful secondary metabolites of fungi that contaminate crops, resulting in detrimental effects on animal and human health as well as animal productivity. Mycotoxin production occurs under favourable conditions that allow fungi to grow on feeds and feedstuffs in the field during harvest, storage, or feed processing and transit (Mwende *et al*., 2016). Although over 450 different mycotoxins have been documented, only a few have been extensively studied due to their toxicological effects and economic importance (Dzuman *et al*., 2015). Mycotoxins such as AFs produced by *Aspergillus* species, FBs produced by *Fusarium* and *Aspergillus* species, T-2, DON, HT-2, and ZEN toxins produced by *Fusarium* species, together with OTs formed by toxigenic spp. of *Penicillium* and *Aspergillus genera*, are the most significant mycotoxins in term of economic and health relevance (Makun *et al*., 2012). Among these mycotoxins, AFs, FBs, DON, ZEN, and OTs are the common toxins in Africa in relative to other mycotoxins as shown in Figure 2.8. While mycotoxins of great concern to dairy cattle include DON, ZEN, T-2 toxin, FBs, AFs, OTs, and ergots.

Mycotoxins are regarded as the most concerning group of fungal metabolites due to their prevalence in agricultural commodities and their high level of toxicity in animals and humans. These toxins can enter the human field chain via two ways: (i) firstly, directly, after human exposure by ingestion of contaminated plants or finished processed food products due to the stability of AFs and their resistance to food processing methods. (ii) Secondly, indirectly from foods such as meat, eggs, milk, and dairy products of animals fed AFs contaminated feeds, via excretion of the hydroxylated derivative of AFB_1 and AFB_2, such as aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2), respectively.

Mycotoxins have been linked to several acute and chronic health effects in humans and animals (Yang *et al*., 2020; Dänicke *et al*., 2021). For example, AFs was detected in tissues of infants with kwashiorkor and Reyes's syndrome and was assumed to be a causative factor of these life-threatening ailments. Reyes's syndrome, a condition characterised by visceral deterioration and encephalopathy, induces swollen of kidney, brain (cerebral oedema) and liver (Cao *et al*., 2020). Mycotoxins may also impair growth development in infants (Sengling *et al*., 2019). According to

Jiang *et al*. (2005), the changes in differential subset distribution and functional alteration of lymphocyte subsets were linked to mycotoxins (AFs) exposure in Ghanaian adults, and he further revealed that AFs may impair human cellular immunity, resulting in decreased infection resistance.

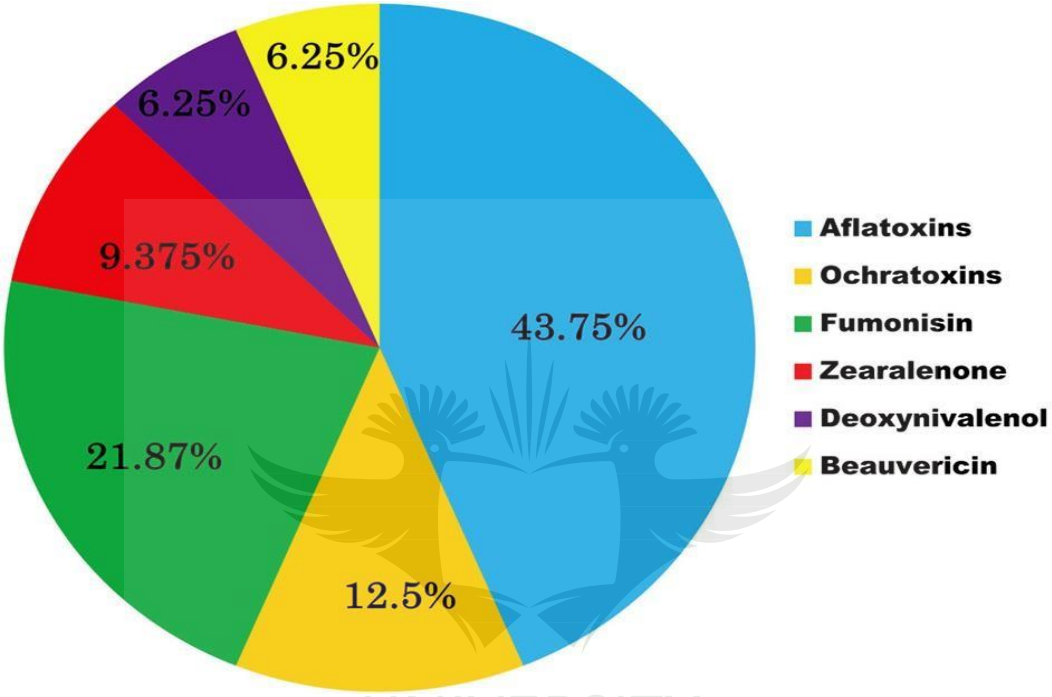


Figure 2.8: Mycotoxins distribution in African countries illustrated by Darwish *et al*. (2014).

2.5.1 Aflatoxins

Aflatoxins are chemical substances produced mainly in nature by many toxigenic fungi, particularly *Aspergillus* spp. They are the most toxic mycotoxin to humans and animals, producing acute and chronic toxicities. The term aflatoxin came into existence in the 1960s, following the epidemic (Turkey X disease) outbreak, which killed over 100,000 birds (turkeys) in England after consuming AF-contaminated groundnut meal (Njobeh, 2009). Similar reports were received from

Uganda, the United States, Kenya, and a few other countries, indicating that this outbreak was not restricted to turkeys (Bedi and Khare, 2012). Due to the severe economic implications caused by this disease, several scientists nationwide began an intensive investigation. In 1961, *A. flavus* was identified as the producer of the toxic metabolites responsible for this chronic disease, and the metabolite was later named ''Aflatoxin,'' meaning *A. flavus* toxins (Sargeant *et al*., 1961). Besides *A. flavus*, several members of *Aspergillus* spp, including *A. parasiticus, A. ochraceoroseus,* and *A. nomius* have been reported to produce AFs (Varga *et al*., 2009; Yunes *et al*., 2020).

Molecularly, there are two groups of aflatoxins: difurocoumarocyclopentenone (AFB_1, AFB_2, AFM_2, AFQ_1, and AFL) and difurocoumarolactone (AFG_1 and AFG_2) (Bennett and Cahill, 2016). Aflatoxins are made up of two furan rings connected by a coumarin moiety. Furofuran rings have been identified as the structures responsible for the toxic and carcinogenic activities when metabolically activated (IARC, 2012). Figure 2.9 shows the chemical structures of AFs. *A. flavus* are notable producers of the B-types AFs (B_1 and B_2), whereas *A. parasiticus* produce the G-types (G_1 and G_2) in addition to the B-types. Taxonomy studies using modern analytical techniques have recently established that *A. flavus* may produce both B and G-types (Gilbert *et al*., 2018; Frisvad *et al*., 2019). Due to its detrimental effects on living organisms, AFB_1 is regarded as the most toxic and studied AF (Sardinas *et al*., 2011; Ráduly *et al*., 2020).

Other notable AFs are AFM_1 and AFM_2; these two AFs are hydroxylated derivatives of AFs (B_1 and B_2) primarily found in tissues and body fluids, including urine and blood, as well as dairy products. AFM_1 is the most common AFB_1 metabolite found in cow milk when dairy animals consumed mycotoxin contaminated feeds (Makun *et al*., 2012; Flores-Flores *et al*., 2015). Several studies have shown that AFM_1 is both mutagenic and teratogenic and has recently been classified as a first group human carcinogen (Palacio *et al*., 2016; Marchese *et al*., 2018).

Although AFs are of global threat, they are widespread in the tropical and sub-tropical regions, where the prevailing humid and warm conditions, mechanical and insect damage of crops, and the prevailing agricultural practices are more favourable to their production than the temperate, cool, or arid climates (Kebede *et al*., 2020). They have been confirmed as natural contaminants of crops such as cereals (Egbuta *et al*., 2015; Echodu *et al*., 2019), peanuts and peanut butter (Mupunga *et*

al., 2014), and ready to eat food (Ezekeiel *et al.*, 2020). These toxins have also been reported worldwide in dairy cattle feeds and feed ingredients (Gizachew *et al*., 2016; Palacio *et al*., 2016; Rodríguez-Blanco *et al*., 2019), dairy cattle milks (Claudious, 2019; Kagera *et al*., 2019), as well as milk products (Iqbal *et al*., 2015; Sumon *et al*., 2021). In research carried out by Ndlovu and Dutton (2013) to determine the mycotoxin encountered in South African's maize silage, an important dairy cattle feed, they reported AFs as the most prevalent mycotoxin, occurring in 97% of the total sample with minimum and maximum concentrations of 0.2 and 67 µg/kg, respectively. Similarly, analysis of 92 commercial compound feed from South Africa showed dairy cattle feed as the most contaminated feed with a 52% incidence rate and mean and maximum concentrations of 14.7 ± 22.8 and 71.8 µg/kg, respectively (Njobeh *et al*., 2012). The authors emphasized that concentrations of 4 of the analysed samples surpassed the regulatory limits (10 µg/kg) set by the South African government for total AFs in dairy cattle feeds. Changwa *et al*. (2018) detected AFB_1 and AFG_2 as the most frequent among the AFs in South African dairy cattle feeds, the minimum and maximum mean concentrations reported were 2.1 and 41 µg/kg, respectively. In recent research to determine the level of mycotoxins in feeds destined for dairy cattle consumption in South Africa, Changwa *et al*. (2021) also reported AFs in 77 feed samples (compound and forages) with a minimum value of 2.2 µg/kg and a maximum level of 30.2 µg/kg, respectively.

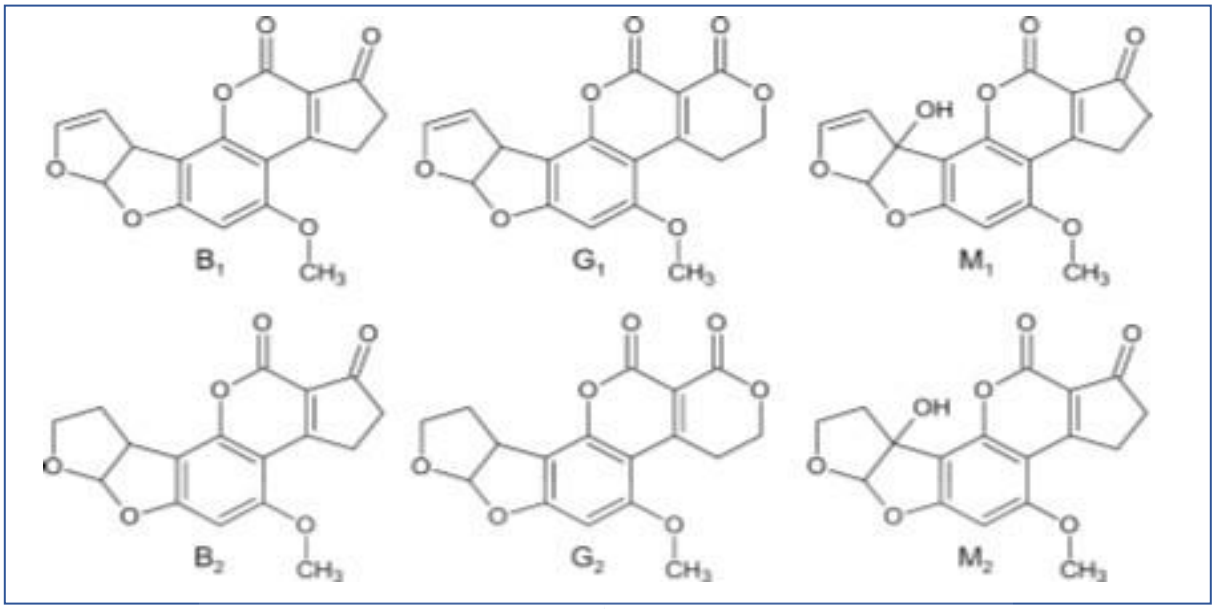


Figure 2.9: Chemical structures of aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, and AFM_2) (Adapted from De Ruyck *et al*., 2015).

Aflatoxins are teratogenic, carcinogenic, and immunosuppressive, and they have been linked to chronic carcinogenicity as well as acute toxicity in animals and humans (Bennett and Klich, 2003). The degree of toxicity and toxicological effects vary substantially depending on the AF type as well as the age, gender, nutritional and health status of the host. Aflatoxin contamination in feeds has been related to liver damage, decreased feed intake, decreased milk output, and increased animal mortality in livestock (Zain, 2011; Flores-Flores *et al*., 2015). Aflatoxicosis in animals have already been described in the field and laboratory. Van Halderen *et al.* (1989) in South Africa observed a field outbreak in which 7 of the total 25 calves given aflatoxin contaminated feeds (11, 790 µg/kg) reportedly died. Aflatoxicosis in dairy cattle has been described experimentally, with symptoms varying from low feed intake, decreased milk production, lameness, hepatoxicity, and immunosuppression to nephrotoxicity (Goncalves *et al*., 2015). Jiang *et al*. (2018) found a significant decline in milk output in cattle fed 75 µg/kg dry matter of AFB_1 for five days. Furthermore, AFB_1 has been demonstrated to inhibit DNA, RNA, and protein synthesis, resulting in immunosuppression and teratogenic consequences (Cavaliere *et al*., 2010; IARC, 2012; Okafor

and Eni, 2018). Lastly, aflatoxin's exposures have also been proven to cause hormonal imbalance in children, resulting in stunting growth in children from Sierra Leone (Jonsyn-Ellis, 2012).

2.5.2 Ochratoxins

Ochratoxins was discovered in 1965 by a group of South African researchers, who extracted ochratoxin A (OTA) from *A. ochraceus* grown on maize meal in South Africa (van der Merwe *et al*., 1965). *Aspergillus* and *Penicillium* spp., mainly *A. niger, A. carbonarius, A. alliaceus, A. ochraceus, A. melleus and P. verrucosum* are the major producers of OTs (Bayman and Baker, 2006) that colonise agricultural commodities. They exist in 3 secondary metabolites forms: ochratoxin A (OTA), ochratoxin B (OTB) and ochratoxin C (OTC) (Figure 2.10). The three forms differ by the fact that OTB and OTC are non-chlorinated and ethyl ester forms of OTA (Bayman and Baker, 2006). Conversion of OTA to OTB occurs through substitution reaction in which the chloride present in the isocoumarin moiety is replaced by a hydrogen atom or to the C type (OTC) via the addition of an ethyl ester to the phenylalanine moiety (van der Merwe *et al*., 1965). Among these three forms, OTA is regarded as the most significant due to its frequent occurrence in crops and toxicity (Duarte *et al*., 2010). Ochratoxin A appears as a colourless crystal under normal light, and however, under ultraviolet light, it fluoresces green and blue.

Due to the chemical stability of OTs, particularly OTA, ordinary food or feed processing measures failed to significantly reduce its presence in foods and feeds. Ochratoxin A is a common contaminant of several agricultural commodities, including cereals (barley, rye, oat, wheat, and corn) (Terzi *et al*., 2014; Neme and Mohammed, 2017; Hassan *et al*., 2018; Tao *et al*., 2018), cocoa products (Anne-Marie *et al*., 2013), coffee (Leitão, 2019), as well as wine and beer (Arrúa *et al*., 2019; Silva *et al*., 2020). It also contaminates dairy cattle feeds such as hay and mixed feed (EFSA, 2004), and dairy cattle milk (Tale Hel Abad *et al*., 2016). Limited information is available in South Africa regarding contamination of dairy cattle feeds and feedstuffs by this toxin. Njobeh *et al*. (2012) reported OTA in 95 compound feed samples obtained from South Africa, OTA was recorded in 16% in cattle feeds with maximum concentration of 17.1 µg/kg. Changwa *et al*. (2018) found no OTA in analysed dairy cattle feeds and feedstuffs from South Africa. However, Changwa *et al*. (2021) later found this mycotoxin in 77 dairy cattle feed samples at a low incidence rate of

3.9% with maximum and mean levels of 187.9 and 85.6 ug/kg, respectively, in contrast to their previous study on OTA in dairy cattle feeds and feed ingredients.

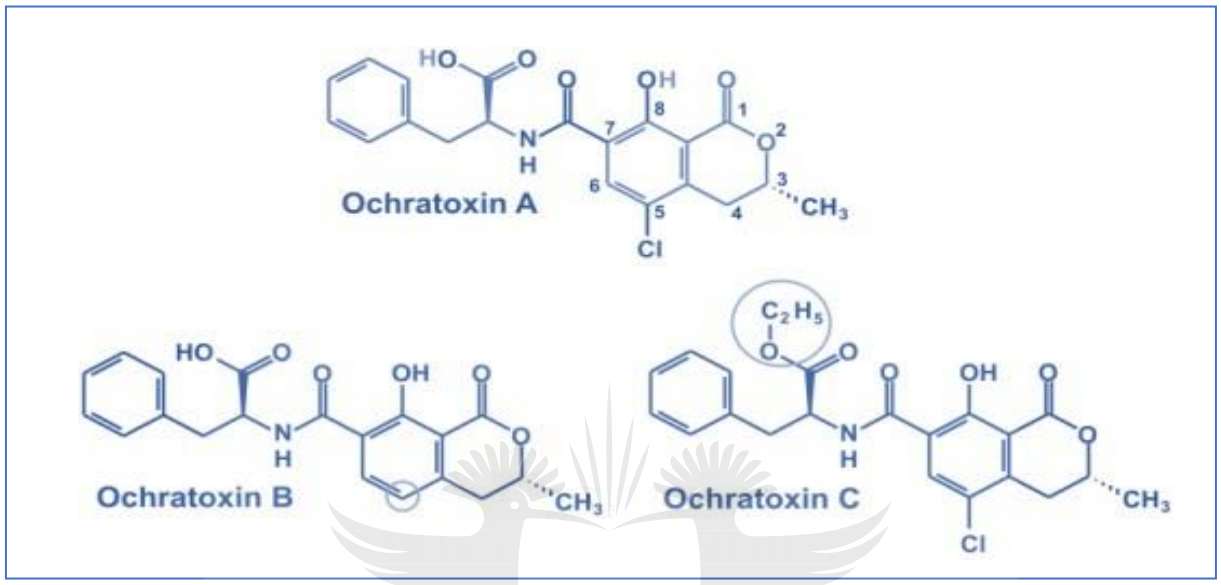


Figure 2.10: Chemical structures of ochratoxins (OTA, OTB and OTC) (Adapted from Kőszegi and Poór, 2016).

Ochratoxin A is nephrotoxic, causing acute and chronic kidney lesions in several animal species, as well as immunotoxic, hepatotoxic, teratogenic, and carcinogenic (Pfohl-Leszkowicz and Manderville, 2007; Liang *et al*., 2015). Furthermore, long-term OTA exposure causes poor growth rates, poor feed conversion and feed refusal in farm animals (Kemboi *et al*., 2020). OTA is distributed primarily to the kidneys, with minimal concentrations in the liver and muscle, and its rate of disappearance from blood is slower than from tissues (Janik *et al*., 2020). Ochratoxicosis, a disease caused by OTA, is a rare occurrence in cattle. This is due to the rumen microbiota's ability to efficiently break down OTA into non-toxic compounds. However, Ribelin *et al*. (1978) observed diarrhoea, anorexia, and a decrease in milk output in dairy cattle administered a high single dose of 13,300 µg/kg OTA with recovery four days after. In many animal species, OTA

poisoning symptoms are believed to be dependent on the dose used, as well as the duration of exposure (Ráduly *et al.*, 2020). The human aspects of OTA exposure are yet to be fully elucidated. Nonetheless, the toxin has been linked to kidney damage, kidney failure and cancer in humans (Heussner *et al*., 2015). The so-called Balkan Endemic Nephropathy was a well-documented example (Barnes *et al*., 1977). For this reason, OTA was designated by the International Agency for Research on Cancer (IARC) as a group 2B human carcinogen in 1993.

2.5.3 Fumonisins

Fumonisins are among the most recent discovered fungal metabolites with high cancer-inducing properties (Bennett and Klich, 2003), first discovered in South Africa in 1988 (Marasas, 2001). They are produced primarily by *Fusarium* spp, of which the major producers are *F. verticillioides* and *F. proliferatum*. Perera *et al*. (2021) later reported that some FBs (FB_2 and FB_4) could also be produced by members of the *Aspergillus* spp., especially *A. niger* and *A. welwitschiae*. At least fourteen FBs are known, of which FB_3, FB_2 and FB_1 are the most naturally occurring ones (Dragon *et al*., 2001). FB_1 is a diester of propane-1, 2,3-tricarboxylic acid and 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyeicosane (Njobeh, 2009), whereas FB_2 and FB_3 are esterified, respectively, at C_{10} and C_5 deoxy analogues of FB_1 (D' Mello, 2003) (Figure 2.11).

These toxins have been reported worldwide in many agricultural products, mostly in maize and maize finished products. The work conducted by Vismer *et al*. (2015) in West Africa to assess FBs contamination in cereals crops revealed the highest contamination in maize with mean level of 228 ± 579 µg/kg), followed by pearl millet and sorghum with mean levels of 18 ± 7 and 131 ± 270 µg/kg, respectively. Despite few reports on mycotoxins in feed globally, FBs contamination of dairy feeds and feedstuffs appears notably. In Spain, high levels of FBs were found in 41% of the total 95 silages for dairy cattle, with concentrations ranging from 469 to 2,565 µg/kg (Ramos *et al*., 2019). The work of Njobeh *et al*. (2012) in South Africa to evaluate the mycotoxins contaminating dairy cattle compounded feeds revealed a maximum value of 2,499 ug/kg, the second highest after chicken feeds (2,999 µg/kg). Likewise, Chilaka *et al* (2012) reported FB_S (FB_1, FB_2 and FB_3) contamination in forty commercial maize samples from Kwazulu-Natal

province in South Africa with 100% contamination. The total FBs recovered ranged from 64 to 1,035 µg/kg, with an average concentration of 455 µg/kg.

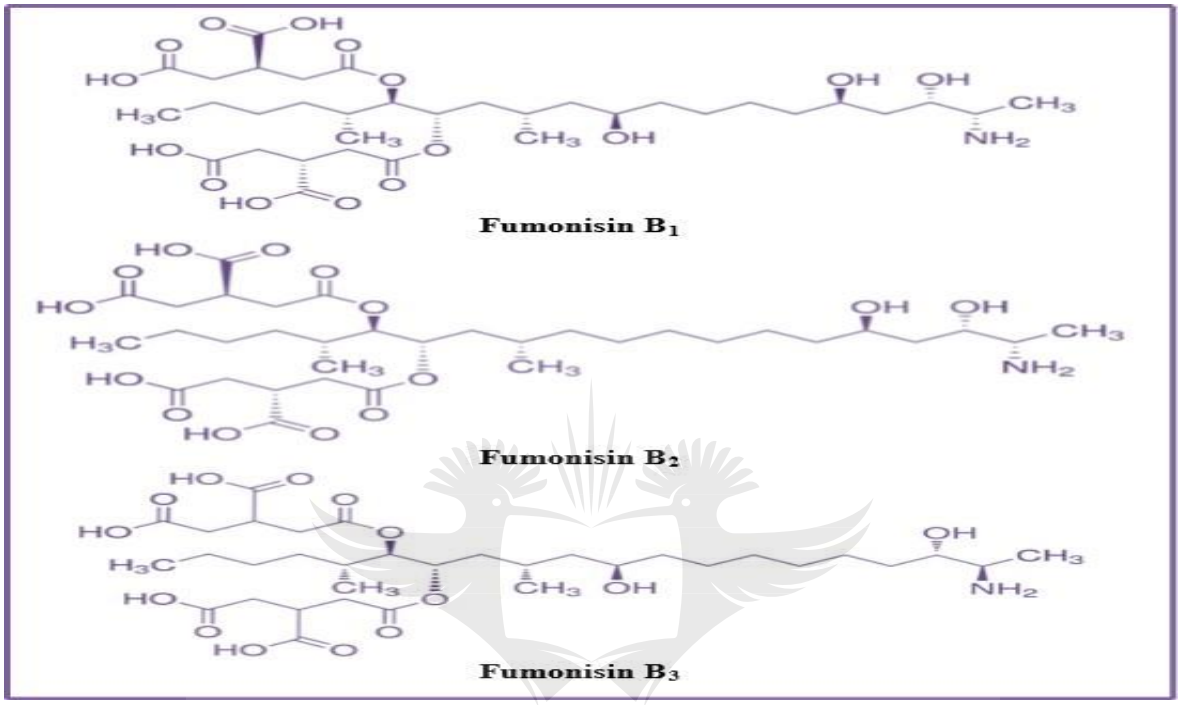


Figure 2.11: Chemical structure of fumonisins (FB_1, FB_2 and FB_3) (Adapted from Phoku, 2014).

Associated health effects of FBs are exhibited in animal and human tissues with lesions found in the oesophagus, gastro-intestinal tract, lungs, liver, and brain. Human consumption of FB_1-contaminated foods has been correlated with increased incidence of upper gastro-intestinal tract cancer in several countries, including China (Misihaivabgwia *et al.,* 2019), northeast Italy (Soriano and Dragacci, 2004) and among black people in Charleston, South Carolina (Sydenham *et al*., 1991). High exposure of human to FB_1 in the Transkei region (now Eastern Cape) in South Africa has previously been linked to the contamination of maize by FB_1 in that area. Cattle are generally resistant to many mycotoxin effects because of the degradation of these toxins by their rumen

microbes, but FBs is hardly degraded in their rumen (Fink-Gremmels, 2008; Gallo *et al*., 2020). Thus, a portion of it consumed by cattle is passed out via faeces. Hence, the gut is overwhelmed by the toxin and this result in significant health issues in cattle, including hepatic damage, reduced feed intake, decreased milk production and reproduction problem (Kemboi *et al*., 2020).

2.5.4 Zearalenone

Zearalenone is a chemical compound formed naturally in crops by *Fusarium* fungi. The first case of ZEN toxicity was described in the early 1920s, following the discovery of hyper-estrogenusm in pigs fed mouldy grains. Zearalenone is commonly produced by an array of *Fusarium* spp., such as *F. culmorum, F. semitectum, F. equiseti, F. verticillioides*, *F. lateritium, F. crookwellense, F. graminearum, F. cerealis* and *F. roseum* (Gajecki, 2002; Chilaka *et al*., 2017). This toxin is an enantiomorph of 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcyclic acid lactone (Liu and Applegate, 2020) (Figure 2.12).

Zearalenone has been reported in a wide range of agricultural commodities, including cereal grains, rice, maize, and other staple foods consumed across SSA (Egbuta *et al*., 2015; Olopade *et al*., 2021). It has also been reported in dairy feeds such as maize silage (Ramos *et al*., 2019) and complete feed (Zain, 2011). Contamination of dairy cattle feeds with ZEN has been reported in South Africa. Njobeh *et al*. (2012) revealed ZEN in dairy cattle feeds at a low incidence rate with (mean: 72 ± 43 µg/kg; maximum: 123 µg/kg). Additionally, Shephard *et al*. (2013) confirmed the incidence of 61 and 32% ZEN in mouldy and good maize within the range of 0.1 to 1,648 and 0.6 to 329 µg/kg, respectively, from the Transkei region, South Africa. A similar report of ZEN in South Africa by Changwa *et al*. (2021) also confirmed its presence in 77 dairy cattle feeds with levels ranging from 96.7 to 1,793.7 µg/kg. Some of the ZEN levels reported by the authors were above the regulatory limits of 500 µg/kg for South African dairy cattle feeds.

The specific physiological pathways of the toxic effects of ZEN in agricultural animals are unknown. Feeds containing about 1,000 µg/kg of ZN increase estrogen receptor expression and decrease follicle integrity when fed to lactating pigs (Schoevers *et al*., 2012). This mycotoxin has a structure like the human sex hormone (17-β-estradiol), which aids its binding to the estrogen

receptors in target cells, resulting in infertility issues in both animals and humans (Adegbeye *et al*., 2020; Wan *et al*., 2021). Swine, poultry, cattle, and experimental animals are the most typically afflicted by this toxin. Additionally, ZEN has recently been classified as a group 3 carcinogen by the IARC Monograph (IARC, 1999). Fungal proliferation and subsequent mycotoxin production are influenced by certain environmental factors, these will be discussed subsequently.

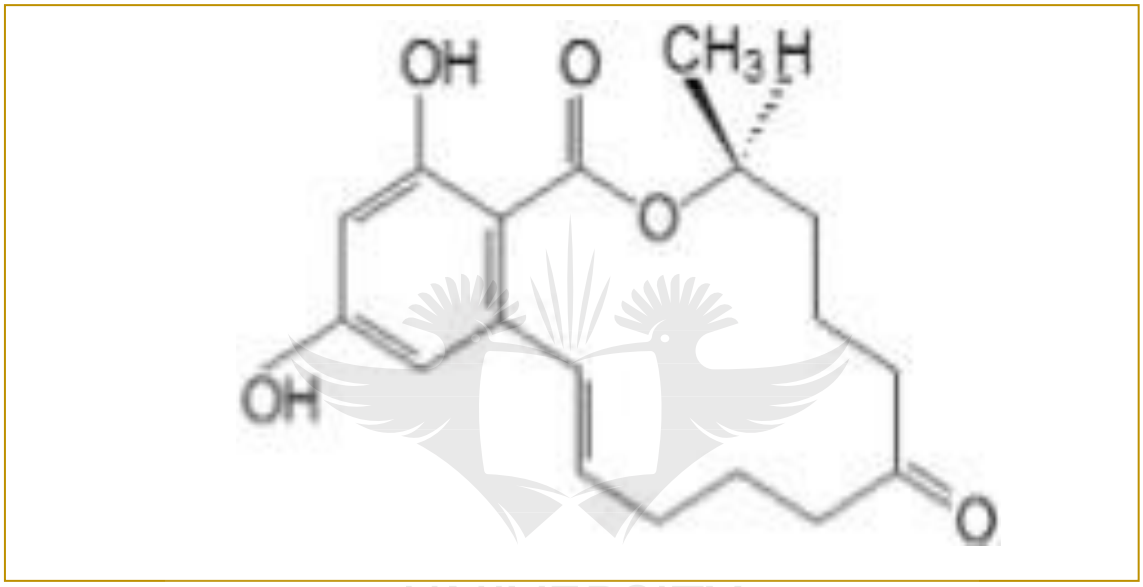


Figure 2.12: Chemical structure of zearalenone (Adapted from Da Rocha *et al*., 2014).

2.6 FACTORS INFLUENCING FUNGAL PROLIFERATION AND MYCOTOXIN CONTAMINATION IN SOUTH AFRICA.

The factors influencing fungal proliferation and mycotoxin production are classified using various categories. Some classifications categorised these factors as chemical, biological, and physical, others as intrinsic and extrinsic, while some classified them as environmental, storage and ecological factors (Zain, 2011; Atanda *et al*., 2013). Regardless of the classification, Lacey (1986) revealed that the amount and type of mycotoxin produced often depend on the fungus, the

substrate, and the environment. In South Africa, we can classify these factors into six types as detailed subsequently without necessarily adhering to any prior categorisation systems.

2.6.1 Climatic conditions

Mycotoxin producing fungi, according to Atanda *et al.* (2013), occur more frequently in the tropic and are well-known as prominent agricultural commodity spoilage agents in these warmer climates. High humidity and temperatures are the two major environmental factors affecting fungal proliferation and mycotoxin production (Wagacha and Muthomi, 2008; Mwende *et al.*, 2016). Temperature's role in fungal survival may be related to its effect on enzyme activity and cell membrane structure (Chin *et al.*, 2010). Although fungal colonisation and mycotoxin production are related, the optimal temperature and humidity required for mycotoxin formation vary depending on the fungus and its attendant toxins (Pitt and Hocking, 2009). It has been affirmed that *Aspergillus* spp. need a higher temperature range (15 to 40 ^{o}C) for growth than *Penicillium* spp. (25 to 30 ^{o}C), however, the optimal temperature range of 37 to 47 ^{o}C is suitable for *Aspergillus* growth and 28 to 30 ^{o}C for most *Penicillium* (Pitt and Hocking, 1997). Unlike *Penicillium* and *Aspergillus* spp., *Fusarium* spp. are psychrophilic, i.e., growing and reproducing under cold temperature (Rico-Munoz, *et al*., 2019).

The optimal temperature needed for mycotoxin biosynthesis ranges between 25 to 33 ^{o}C. Abarca *et al*. (2003) revealed that *A. ochraceus* needs a maximum temperature of 30 ^{o}C to produce OTA. Likewise, Bhat *et al.* (2010) found that some *Fusarium* genera produce trichothecenes at lower temperatures than most mycotoxins. Despite the fact that aflatoxins can be produced at a variety of temperatures, an ideal range of 25 to 35 °C has been confirmed for their maximum production (Siciliano *et al*. 2017). However, more AFB are produced than AFG at high temperature, but the production of both toxins is said to be the same at low temperature (Matumba *et al.*, 2015). It has also been established that 70 to 90% relative humidity is optimum for fungal growth and most mycotoxin formation (Wu *et al*., 2011). Ding *et al* (2015) recently confirmed that a 95% relative humidity significantly boosts AFs production. These conditions are similar to the ambient climatic conditions in many African countries and thus, account for the continent's high prevalence of mycotoxins in most of her agricultural products.

Drought conditions also promote plant stress, exposing them to fungal infection and mycotoxin contamination. The impact of climate change was observed in Hungary, wherein, the increase in AFs contamination was attributed to climate change conditions (Dobolyi *et al*., 2013). A similar example was reported in Serbia, where initially no contamination was detected, but the 2012, hot and dry weather resulted in 69% of maize being contaminated with AFs (Medina *et al*., 2015). In the Northwest province of South Africa, Omotayo *et al*. (2019) found higher mycotoxin concentrations in summer than winter ginger.

2.6.2 Pests and insects

Other factors favouring colonisation of food and feed by toxigenic fungi, as well as mycotoxin production in them, are insects and other pests (Jeyaramraja *et al*. 2018). Insects are the major vectors of deterioration and sources of grain and seed losses. Insect infestation of cereals reduces their quality, grade, and market value, causing massive economic losses (Kumar *et al*., 2021). Pests and insects also cause infectious wounds on crops through their feeding habits (Munkvold, 2003), and these wounds, according to Kinyungu (2019), cause stress to the plant, thereby exposing them to contamination by toxigenic fungi. Pest and insect infestations of crops are caused mainly by poor harvesting and improper storage conditions, with the level of fungal infection and mycotoxin contamination influenced by the extent of damage caused by the pests or insects. It has also been confirmed that insect damage to maize can trigger *Fusarium* contamination (Avantaggio *et al*., 2002). Phoku *et al*. (2014) isolated several toxigenic fungi from houseflies in South Africa. The fungal species recovered from these insects were tested positive for some significant mycotoxins (ZEN, DON, FB_1, AFB_1, AFB_2, AFG_1, AFG_2, and OTA)

2.6.3 Water activity

Water activity is the most crucial environmental component influencing the growth of microbes like fungi and, as a result, influence the stability of stored farm products. Fungi require moisture for their growth and formation of secondary metabolites, and the amount needed, however, varies from species to species. These microorganisms require water for nutrient uptake through the cell wall and membrane, to release extracellular enzymes and for metabolism. Fungi are classified into

two groups based on their optimal moisture ranges for growth. These include xerophilic fungi (those that thrive at very low a_w), such as sebi wallemia and *Eurotium* spp., and hydrophilic fungi (those that grow at extremely high a_w) including *Ulocladium, Chaetomium,* and *Stachybotrys* (Steel, 2009).

2.6.4 Presence of Oxygen

Most fungi are aerobic, requiring oxygen in some stages of their life cycle, whereas some species can grow without oxygen with the formation of organic acids and ethanol. Also, mycotoxin production by various fungi can be affected by the absence or presence of oxygen in the environment (Pitt and Hocking, 2009). For instance, Northolt (1979) reported that penicillin acid and PAT synthesis decrease at low oxygen concentrations, while the growth of fungi is noticeably not influenced. Mycelial growth and spore formation of fungi are sensitive to both low and high oxygen concentrations in different ways. According to Pitt and Hocking (1997), *Aspergillus* formation is limited under very low oxygen concentration (1 < %).

2.6.5 Pre-harvest, time of harvesting and post-harvest handling conditions

Other vital elements impacting mould growth and toxin production are pre-harvest, time of harvest and post-harvest managements. Cole *et al.* (1995) identified soil type, genotype, plant density, and drought as essential factors influencing the likelihood of pre-harvest contamination. In contrast, Abbas *et al*. (2002; 2007) concluded that high nighttime temperatures promote mould growth and mycotoxin production when a plant is deprived of its natural source of energy and unable to repel fungal attack. Harvesting is the first stage of production, and it is at this point, the moisture content of the plants becomes critical for crop management and protection from field fungal species. Early harvesting has been demonstrated to reduce fungal colonisation of plants in the field. Kaaya *et al*. (2006) showed that AFs levels in maize increases four folds due to three weeks delay in maize harvest and more than seven times by the fourth week. Nevertheless, early harvesting of crops must be followed by adequate drying to acceptable moisture levels in order to prevent possible fungal growth and subsequent mycotoxin production (Atanda *et al.,* 2013). Fungal and mycotoxin contamination of crops can also occur due to improper post-harvest handling. As a

result, post-harvest transit of agricultural products can be problematic because these crops pass through multiple intermediaries, including traders and intermediate processors, who may be located in another region (Atanda *et al*., 2013).

2.6.6 Storage facilities and conditions

The presence of fungi in food and feed products may be caused by the storage methods applied. As such, improper storage of agricultural commodities may result in deterioration of these substrates by a group of fungi known as storage fungi, which infest plant products during storage if storage conditions are not adequately controlled (Atanda *et al*., 2011). Food and feed spoilage during storage is affected by specific conditions such as nutrient composition in the substrates, storage temperature, moisture content of the substrates, as well as biotic factors, including insects (Atanda *et al*., 2011). Maximum growth of storage fungi, especially *Aspergillus* spp. can happen when the temperature is about 30 ^{o}C, and the relative humidity between 80 and 90%, respectively (Pardo *et al*., 2005). To retain crop quality during storage, it is critical to reduce or avoid biological activity by drying to a moisture content of less than 10%, and to limit activities of insects, which can increase moisture levels (Turner *et al*., 2005). There is little information available on the method of storage of farm products by farmers in South Africa. The farmers stored their cereals and other farm products in an unhygienic environment which encouraged the growth of mycotoxigenic fungus, increasing the danger of mycotoxin contamination (Phokane *et al.*, 2019).

2.7 FEED SAFETY AND ECONOMIC IMPACT OF MYCOTOXINS ON DAIRY CATTLE

Ensuring food safety is a difficult task because food contamination can happen at any stage along the food chain, from primary producers to ultimate consumers, that is, from farm to plate. It is imperative to understand that feed safety is critical to food safety, i.e., it is an essential measure for quality food and feed availability worldwide, where fungi and mycotoxins are causing significant losses to agricultural products, adverse effects on health and economic welfare, and, in the worst-case scenario, direct loss of human life due to deaths (Udomkun *et al*., 2017; Omotayo *et al*., 2019). The Technical Centre for Agricultural and Rural Cooperation (CTA) issued a warning

that mycotoxin poses a threat to African food security, undermining the UN's fundamental goal of boosting nutrition, establishing food security, and generating healthy agro-economic growth (AUC-PACA and CTA, 2016).

Approximately 25% or more of global crops is lost annually due to mycotoxin contamination, which severely impacts feed and food availability, and animal productivity (Enyiukwu *et al*., 2014; Gbashi *et al*., 2018). Farmers' revenues are thus reduced due to product rejection or lower market value, diminishing their profit margin. Economic losses caused by mycotoxicosis are challenging to quantify in developing nations, especially Africa. While developed countries incur solely economic losses because of mycotoxin-contaminated feed or food trade challenges, developing countries face both health issues and economic losses because of this contamination (Gbashi *et al*., 2018). Developing an economic model to assess the global impact of mycotoxin has proven difficult, and as a result, most economic impact studies focused on a specific aspect of mycotoxin contamination or exposure (Hussein and Brasel, 2001). Some of the criteria used in evaluating the economic impacts of mycotoxins on animals and humans include loss of agricultural products, human and animal fatalities, veterinary and health care costs, research costs, and regulatory costs directed towards mitigating the impacts and severities of the mycotoxin problems (Zain, 2011).

Contaminated feed poses significant economic and food security issues in the dairy industry. The economic impacts arose from the actual market costs associated with lost trade or reduced profits caused by tainted products, as well as reduced dairy productivity, death of dairy animals, and increased treatment and mycotoxin mitigation costs (Ghashi *et al*., 2018; Kemboi *et al*., 2020). This has a negative influence on all the stakeholders involved in dairy production, such as dairy farmers, feed producers, milk processors as well as milk and dairy products consumers (Rodrigues *et al*., 2011). The economic impact of mycotoxins on dairy cattle is not well understood in Africa. It was revealed in Kenya that 61.4% of AFB_1 contaminated feed were above the 5 ug/kg limit level set by FAO/Kenya. This amounts to a prospective annual economic cost of 22.2 billion US dollars for dairy feed producers, with additional 37.4 million US dollars due to losses sustained by dairy farmers yearly because of reduced milk yield due to ingestion of AFB_1 contaminated feed by dairy cattle (Senerwa *et al*., 2016). In the same study, 10.3% of milk analysed was contaminated with AFs, with levels exceeding the FAO/WHO regulatory limits (0.5 µg/kg), which would cost dairy

milk producers around 113.4 US dollars annually if legislation was followed. Since contamination of crops used as dairy cattle feed by fungi and mycotoxins may occur in the field during the vegetation, harvesting, processing, and transportation or during feed storage, together with their negative impacts on humans, animals, and the economy. It is, therefore, essential to monitor and control fungal and mycotoxin contamination in dairy cattle feeds to reduce their levels in human diets.

2.8 FUNGI AND MYCOTOXIN CONTROL

Fungal contamination of animal feeds not only reduces feed quality but also results in mycotoxins production. The harmful impacts of these toxins on human health and the economy has prompted researchers into strategies to eliminate, deactivate, and reduce their bioavailability in human and animal diets (Goncalves *et al*., 2015). Mycotoxin removal from agricultural products can be accomplished through biological, physical, and chemical means (Corassin *et al*., 2013; Azam *et al*., 2021). The biological measures are based on the action of microbes such as yeast, algae, mould, and bacteria on mycotoxins. These microbes compete with the toxins for the available nutrients and space (Fazeli *et al.*, 2009). Contamination of feeds and feeds ingredients with toxigenic fungi and mycotoxins can also be prevented or mitigated by good farming practices such as crop rotation and irrigation, proper storage method, genetic engineering (using high fungal resistant and insect resistant crop varieties) such as the use of atoxigenic fungus like the case of atoxigenic strains of fungus in the field to outcompete with toxigenic strains of *A. flavus* (Agbetiameh *et al*., 2019; Bandyopadhyay *et al*., 2019). Another biological means of reducing mycotoxins in feeds is fermentation. Several studies carried out at the University of Johannesburg, South Africa published in the literature have identified fermentation as an effective method of reducing/degrading mycotoxin levels in crops by altering the chemical structure of the mycotoxin (Adebiyi *et al.*, 2019; Adebo *et al*., 2019).

Numerous chemical substances like acids, aldehydes, oxidising agents and alkalis, and several gases are proved to inhibit toxigenic fungal proliferation and mycotoxins formation (Kumar *et al*., 2021). Ozone was discovered to be the most effective gas for enhancing AFs degradation on cereals and legumes via an electrophilic attack on the furan ring's carbon bonds of the toxin (Jalili,

2016). Other chemicals capable of reducing mycotoxins in feeds and feed ingredients are calcium hydroxide, formaldehyde, sodium bisulfite, sodium hypochlorite, and absorbents (Carvajal and Castillo, 2009). These chemicals can bind mycotoxins firmly in feeds, preventing them from being absorbed by the digestive tract of animals (De Oliveira and Corassin, 2014). Thermal inactivation, ionisation radiation, roasting, solvent extraction, and other cooking methods are the physical methods used to decontaminate mycotoxins in agricultural products (Peng *et al*. 2018). About 70 and 79% reduction in AFB_1 and AFG_1 concentrations were noticed after roasting some seed samples at 150 ^oC for 15 min (Jaliali, 2016).

2.9 CONCLUDING REMARKS

From the literature reviewed, it is noticed that animal feed safety is constantly jeopardised by fungi and mycotoxins, particularly in SSA countries like South Africa wherein, the prevailing humid and warm conditions, mechanical and insect damage of crops, improper storage facilities and poor storage conditions coupled with bad prevailing agricultural practices favour fungal proliferation and mycotoxins production. The predominant toxigenic fungal genera contaminating foods and feeds in these regions are mainly *Aspergillus, Fusarium* and *Penicillium.* These mycoflora produce toxic secondary metabolites, including AFs, OTA, DON, FBs, and ZEN. Contamination with fungal toxins have adverse impacts on humans and animals and causes worldwide economic losses. It is, therefore, crucial to assess the safety level of dairy cattle feeds with regards to fungal contamination and mycotoxin production as proposed in the case of Limpopo and Free State provinces of South Africa looking at the contamination at varying seasons.

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1 SAMPLE COLLECTION AND PREPARATION

Various dairy cattle feeds and feed ingredients were selected from 21 smallholder dairy cattle farms in Free State and Limpopo provinces of South Africa between 2018 and 2019. The number of feeds collected from each farm ranged from 1 to 4, depending on the type of feed available (Appendix A, Table 1). The storage systems employed by the farmers in preserving their feeds include keeping in a storeroom, bags, and containers with about 16/21 (76 %) of farmers storing their feeds for less than 1 month, 4/21 (19 %) kept their feeds between 3 – 6 months, while only 1/21 (5 %) stored their feeds for more than 6 months (Appendix A, Table1).

3.1.1 Study areas and criteria for selection

The two agroecological distinct provinces of South Africa chosen for this study were Free State and Limpopo. Free State is located in the central part of the country and has subtropical, cooler arid to semiarid environment, while Limpopo province is located in the country's far north, with warmer arid to semiarid or sub-humid tropical climates**.** Registered active smallholder dairy cattle farmers who are beneficiaries of Agricultural Research Council (ARC) developmental programmes in Phutaditiaba district (Free State) and Vhembe as well as Sekhukhune districts (Limpopo) were selected for this study. The two provinces were therefore chosen based on variations in agro-ecological zones, the vast number of smallholder dairy farms situated there, as well as feed availability.

3.1.2 Sample collection

A total of 70 dairy feeds and feedstuffs consisting of silages, lucernes, pellets, grasses/hays, soybeans, total mixed rations (TMR) and others including maize stover, dairy concentrate, molasses and ramilick were donated by smallholder dairy cattle farmers from Free State and

Limpopo provinces of South Africa over two seasons (summer and winter). The samples were classified into 7 groups as presented in Table 3.1.

Table 3.1: Groups of dairy cattle feeds and feedstuffs collected from smallholder dairy cattle farms, South Africa

	Free State		Limpopo			
Feed type	**Harrismith**	**Phuthaditjhaba**	**Jane Furse**	**Groblersdal**	**Njakajanka**	**Total**
Grasses	**1**	**1**	**3**	**1**	**2**	**8**
Lucerne	**2**	**2**	**4**	**1**	**2**	**11**
Pellet	**1**	**-**	**-**	**6**	**5**	**12**
Soybean	**1**		**4**			**5**
Silage	**3**	**-**	**-**	**-**	**1**	**4**
TMR	**17**	**5**	**-**	**-**	**-**	**22**
Others[a]	**1**	**6**		**-**	**1**	**8**
Total	**26**	**14**	**11**	**8**	**11**	**70**

a = dairy concentrates (4), maize stover (1), molasses (2) and ramilick (1).

3.1.2 Sample preparation

About 300-500 g/ samples were collected and put into sterile plastic bags, kept in cooler boxes, and conveyed to the University of Johannesburg, where they were stored immediately at -4 ^{o}C until fungal enumeration. Each sample was thoroughly mixed to obtain a representative sample. In the laboratory, samples were finely ground with the help of a sterile laboratory blender (LBIOG, ITM Instrument, Alberta, Canada). A 70% ethanol was used to sterilise the blender after grinding each sample. The milled samples were kept at -8 ^{o}C before analysis.

3.2 METHODOLOGY

3.2.1 Fungal isolation

Fungal isolation and enumeration were done as described by Ekwomadu *et al.* (2018) with some modifications. Briefly, 1 g of each blended sample was weighed into a sterile test tube filled with 9 mL of sterilised Ringer's salt solution, vortexed and serially diluted to 10^{-6}. An aliquot of 1 mL of each sample was inoculated in triplicate on solidified Malt Extract Agar (MEA), Czapek Yeast Extract Agar (CYA), and Potato Dextrose Agar (PDA) (Merck KGaA, Darmstadt, Germany) using spread plate technique. To prevent bacterial growth, all petri dishes were supplemented with 100 mg/L each of streptomycin and chloramphenicol. The plates were incubated for 5 to 7 days at 25 ^{o}C. Thereafter, fungal colonies were examined and counted using a colony counter (Gallenkamp, UK). The total and mean fungal loads were counted and expressed in colony forming units per gram of sample (CFU/g) (Pitt and Hocking, 2009).

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

3.2.2 Fungal identification

3.2.2.1 Morphological characterisation

Thereafter, each of the different colonies were sub-cultured on a solidified CYA for *Aspergillus*, PDA for *Fusarium* and MEA for *Penicillium* under aseptic condition. Culture plates were sealed with parafilm and incubated for 5 to 7 days at 27 ^{o}C. Pure colonies were removed and mounted on microscopic slides, stained with lactophenol blue solution, covered with cover slides, and examined under the microscope (Olympus CX40, Micro-Instruments News Zealand, Ltd). The macro- and microscopic identification of the genera *Fusarium* were done in accordance with the

taxonomic keys and guides described by Leslie and Summerell (2006). *Aspergillus, Penicillium*, and other fungal genera were identified according to Klich (2002) and Pitt and Hocking (2009).

3.2.3 Molecular identification

3.2.3.1 DNA extraction

In a situation where the morphological characteristics of individual fungal isolates using the conventional method were insufficient for clear identification, molecular analysis was performed to determine the fungal identity. To accomplish this, genomic DNA was extracted from each fungal culture using a Fungal/Bacteria DNA extraction kit (Zymo Research, D6005, California, USA), following the instructions described by the manufacturer. Briefly, isolates were sub-culturedon PDA plates, and pure mycelia from the 5 to 7 days old cultures were harvested for genomic DNA extraction. Approximately 150 mg of the mycelium was mixed with 700 µL lysis solution contained in a 1.5 mL ZR Bashing Bead^{TM} lysis tube. The extracted DNA was quantified with a ND-1000 spectrophotometer (NanoDrop Technologies) and adjusted to a working concentration of about 50 ng/µL.

3.2.3.2 Polymerase Chain Reaction (PCR) analysis

Polymerase Chain Reaction (PCR) was done after DNA extraction to amplify a DNA fragment of interest within the Internal Transcribed Spacer (ITS) region. An amplicon of about 450 bp was obtained from the genomic DNA of the isolates by using the primer combinations ITS-1; 5'- TCC GTA GGT GAA CCT GCG G - 3' (forward) and ITS-4; 5'- TCC TCC GCT TAT GC-3' (reverse) (White *et al.*, 1990). The PCR was done using the Fermentas 2 X PCR mix (Fermentas Life Science, Lithuania). PCR mix for each sample included 25 µL of 2 x PCR mix, 1 µL of each primer (ITS1 and ITS4), 1 µL of DNA sample, and constituted to a final volume of 50 µL with nuclease-free water. A negative control containing all the reagents except the DNA was also prepared. PCR was performed in an Eppendorf 96-well Thermocycler (Eppendorf, USA) with initial denaturation of DNA set at 95 ^{o}C for 3 mins, 35 cycles denaturation at 94 ^{o}C for 1 min, an annealing step at 55 ^{o}C for 45 secs and extension of primer at 72 ^{o}C for 1 min. This was followed by a last elongation period at 72 ^{o}C for 5 mins.

3.2.3.3 Agarose gel DNA electrophoresis

Successful PCR amplifications were confirmed by staining 4 µL of PCR product with 2 µL of GelRed (Biotium Inc.) nucleic acid dye and running the mixture on 2% agarose gel. A DNA molecular ruler (100 bp ladder; Fermentas O'Gene Ruler) was included in the mixture to determine the base-pair length. After that, generated bands on the gels were visualised with Gel IX imager 20 - 2.8 M Pixel (Bio Olympics, CA, 33 USA) ultraviolet (UV) transilluminator with a wavelength of 312 nm. The PCR products were purified using a DNA ZR-96 sequencing clean up kit (Applied Biosystems, Foster City, CA, USA).

3.2.3.4 DNA sequencing

Purified products were sequenced in both directions (forward and reverse) using the PCR primers and the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing of the amplified ITS region was done on an Applied BiosystemsTM 3730 x l DNA Analyser (ThermoFisher Science, CA, USA). Purification of sequencing products were perfomed using DNA ZR-96 sequencing clean up kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instruction. The forward and reverse sequences were assembled using SeqMan Pro v. 15 (DNASTAR).

3.2.3.5 Phylogenetic analysis

The DNA sequence for each fragment was edited using MEGA V.5.2. Obtained sequences were then blasted against the Gen Bank (http://www.ncbi.nlm.nih.gov/) with BLAST 2.2.31 according to Altschul *et al*. (1997) to confirm the presumptive identity of isolates using similarity index score obtained from blast results. A data set was generated by obtaining the sequence of closely related species to those from this study in a Gen Bank. These sequences were aligned using the online alignment Muscle 3.8.31 (BioNJ) phylogeny.fr (www.phylogeny.fr/simple phylogeny.cgi), after which alignments were checked manually. Thereafter, phylogenetic trees were generated using TreeDyn 198.3 (BioNJ) (www.phylogeny.fr/simple phylogeny.cgi). The phylogenetic relationship in this study was derived from Neighbour-Joining analysis. The bootstrap consensus tree using 1000 bootstrap replicates was constructed in accordance with Felsenstein (1985). However,

branches corresponding to partitions reproduced lower than half (50%) bootstrap replicates were collapsed, with the percentages of the replicate trees given as bootstrap values over the branches. The phylogenetic trees obtained were used to confirm the evolutionary relationship between the isolated fungal species from this study and their relatives in the Gen Bank.

3.2.4 Toxigenicity screening

Aspergillus, Penicillium and *Fusarium* isolates previously recovered from the dairy cattle feed samples were examined for their potentials in producing mycotoxins, such as aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2), ZEN, DON, and OTA. Pure isolates were sub-cultured unto Petri dishes containing solidified CYA, supplemented with streptomycin and chloramphenicol to inhibit bacterial growth. After that, the plates were incubated at 27 ^oC in darkness for three weeks. *Penicillium* and *Aspergillus* toxins were extracted from the solid culture employing the agar plug technique described by Njobeh *et al*. (2009). Briefly, 1 g of pure culture, including the medium, was plugged from each colony's inner, middle, and outer area into an amber vial filled with 4 mL of dichloromethane with a sterile cork borer. The solution was vortexed for 2 mins, left for 60 mins, and further filtered through a 0.22 µm Milex syringe filter unit. The filtrate was collected in a screw-cap amber vial (1.5 mL). To enhance drying, the vials were placed on a heating block set at 60 ℃ under a stream of nitrogen gas and kept at 4 ^oC prior to analysis.

Fusarium toxins were also extracted from the cultures, according to Adekoya *et al*. (2018). Ten grams of each isolate, including the medium, was plugged into a 250 mL conical flask and 50 mL of acetonitrile: water (60/40, v/v) were added. The mixture was placed on a shaker for 60 mins and passed through a Whatman #4 filter paper (Merck, Johannesburg, SA) with the pH adjusted to 6.2 ± 0.3 using 1 M H_2SO_4. The filtrate was further transferred into a separation funnel (250 mL) and extracted three times with 25 mL dichloromethane. Acetonitrile (25 mL) was added to the content previously extracted with dichloromethane, passed through a bed of sodium sulphate anhydrous to remove moisture, and dried over a stream of nitrogen gas. The content was kept at 4 ^oC until analysis.

3.2.5 Mycotoxin confirmation

3.2.5.1 Confirmation by thin-layer chromatography (TLC)

Mycotoxins produced by the fungi extracts were confirmed by two-dimensional thin-layer chromatography (TLC) as described by Patterson and Robert (1979). The extracts were dissolved with dichloromethane (200 µL), mixed by vortexing, and 20 µL of the extract's solution was spotted about 15 mm above the origin of a two-dimensional aluminium backed TLC plate (Silica gel, Sigma-Aldrich, Germany). The same procedure was also performed for the mycotoxin standards (AFB_1, AFB_2, AFG_1, AFG_2, OTA, DON, and ZEN), for comparison and as reference. The plates were dried at room temperature and 10 mL of mobile phase solvent [dichloromethane: ethyl-acetate: propane-2-ol (DEP), (90:5:5, v/v/v)] and [toluene: ethyl-acetate: formic acid (TEF), (6:3:1, v/v/v)] were prepared and transferred to two different chromatographic tanks.

To enhance the saturation of the tanks by the solvent systems, the tanks were left for about 30 mins, after that, the plates were placed in the first chromatographic tank (DEP), with the origin in the bottom left-hand corner. The plates were withdrawn from the tank before the solvent over-runs and air-dried. After drying, the plates were transferred into the second tank (TEF) at a right angle to the first run, with the origin now at the bottom right-hand corner. The solvent was also allowed to run to the top of the plate. The TLC plates were then removed and dried at room temperature, including that of the standards. The fluorescing colours of the spots produced were viewed under short and long wave ultra-violet (UV) light at wavelength 254 and 365 nm (San Gabriel, USA). To confirm the identity of mycotoxins on the plates, some of the plates were then sprayed with specific reagents for mycotoxins, such as aluminium chloride ($AlCl_3$) solution for zearalenone. To aid in the identification of toxins present, the retardation factor (R_F) for each spot on the TLC plate was determined and compared with those of the mycotoxin standards. Following TLC analysis, all extracts were dried under a stream of nitrogen gas, the vials were placed on a heating block set at 60 ^oC and kept at 4 ^oC for future analysis.

$$R_F = \frac{\text{Distance from the origin to the centre of the substance spot (mm}}{\text{Distance from the origin to the solvent front (mm)}}$$

3.2.5.2 Quantification by liquid chromatography with tandem mass spectrometry (LC-MS/MS).

After TLC analysis, all extracts were reconstituted with 1,500 µL LCMS grade methanol. A 750 µL aliquot of each of the extracts was pipetted into a screw-capped amber vial and diluted with an equal volume of dilution solvent (methanol: acetonitrile, 1:1 v/v), vortexed, and 5 µL was injected into LC-MS/MS. Mycotoxins produced by *Aspergillus* and *Fusarium* isolates were detected and quantified using a Shimadzu LC-MS/MS 8040 instrument (Shimadzu Corporation, Tokyo, Japan) which was equipped with a LC-30AD Nexera chromatograph connected to a SIL-30 AC Nexera autosampler and a CTO-20 AC Prominence Column Oven. The chromatographic separation of analytes was done by RaptorTM ARC-18 (2.7 UM, 2.1 X 100 mm) column (Restek Corporation, Pennsylvania, USA), thermostated at 40 °C. Elution was carried out in binary gradient mode consisting of Solvent A (0.1 % formic acid in deionised water) and solvent B [0.1 % formic acid in acetonitrile and methanol (50:50, v/v)]. Mobile phases A and B were pumped at a constant flow rate of 0.2 mL/min and a maximum pressure limit of 400 bar. The gradient elution programmes established was as follows: 0.1 min at 10 % mobile phase B, linearly increasing mobile phase B to 95% at 8.4 mins and kept constant for 3 mins. The column was allowed to re-equilibrate for 1 min with 10% mobile phase B before proceeding to the next run, which took 4.5 min bringing the total analytical run duration to 17 min.

Analytes were detected and quantified using a Shimadzu 8040 triple-quadrupole MS 8040 (Shimadzu Corporation, Kyoto, Japan) operated in positive ionisation mode with an electron spray ionisation (ESI+) source. The following instrumental parameters were applied: interface nebulising gas flow rate was set at 3 L/min, 250 °C desolvation line (DL) temperature, 400 °C heat block temperature, and drying gas flow rate was set at 15 L/min. Data were obtained by the multiple reaction monitoring (MRM) method operated using optimised MS conditions for the analytes. Table 3.2 shows information about the precursor and product ions of the mycotoxins and

other acquisition parameters. Data were accessed and processed using Shimadzu LabSolutions software.

Table 3.2 MS conditions and MRM transitions of the determined mycotoxins

S/No	Mycotoxin	Precursor ion (mz)	Products ion (mz)	Q1 Pre Bias (V)	Collision energy (CE)	Q3 Pre Bias (V)
1	AFB_2	315	259.10	-22	-31	-25
			287	-23	-26	-30
2	AFB_1	313	241	-22	-41	-23
			285.1	-22	-24	-29
3	AFG_2	331	245.1	-12	-32	-24
			313	-12	-24	-20
4	AFG_1	329	243	-12	-28	-23
			313.1	-16	-24	-14
5	OTA	403.8	239	-15	-27	-24
			221	-12	-38	-21
6	DON	297.10	231	-21	-13	-26
			249.10	-14	-12	-25
7	ZEN	319.1	185	-12	-27	-30
			187.1	-15	-21	-19

Aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), ochratoxin A (OTA), deoxynivalenol (DON) and zearalenone (ZEN).

3.2.5 Method validation

The method performance was validated by evaluating various parameters established by the European Commission (EC 2006). A multi-mycotoxin analytical method for CYA was validated using spiked blank CYA media samples. Validation parameters evaluated included linearity, matrix effects, the limit of detection (LOD) and the limit of quantification (LOQ), and recovery. To assess matrix effects on the analysed samples, both neat standard curves and matrix-matched calibration curves were constructed. Matrix-matched calibration and neat standard curves consisted of seven mycotoxins. The matrix-matched calibration curves were utilised for the quantification of the mycotoxin levels in the samples. Linearity was determined using matrix-matched calibration curves (MMC) by spiking the blank medium (CYA) at seven concentrations. Calibration curves were constructed by plotting the analyte peaks areas (y) versus the analyte concentrations (x). Linear regression was used to fit the calibration curve. The coefficient of determination (R^2) and retention times (RT) for each mycotoxin were also evaluated. The LOD and LOQ were estimated using MMC. LODs were determined as the concentration corresponding to three times the ratio of the standard deviation of the residual divided by the slope (Equation 1), while LOQs equalled the concentration corresponding to ten times the ratio of the standard deviation of the residual divided by the slope (Equation 2) (Shrivastava and Gupta, 2011). All detected analytes were quantified by comparing their peak area on the calibration curve of the equivalent mycotoxin standard to their peak area on the calibration curve of the corresponding mycotoxin standard. The apparent recovery for each mycotoxin was obtained by spiking blank samples at 100 µg/kg (high) and 50 µg/kg (low), and through the comparison of spiked concentration and observed concentration after extraction according to Equation 3 (Tebele *et al.*, 2020).

$$\text{LOD} = 3.3 \times \frac{\text{residual standard deviation of the regression line}}{\text{slope}} \quad (1)$$

$$\text{LOQ} = 10 \times \frac{\text{residual standard deviation of the regression line}}{\text{slope}} \quad (2)$$

$$\text{Recovery} = \frac{\text{measured concentration}}{\text{spiked concentration}} \times 100 \quad (3)$$

3.3 DATA ANALYSIS

Fungal concentrations were determined for all the feed samples by dividing the total number of CFU by the plate volume, and the colonies expressed in CFU/g. Data were analysed using IBM Statistical Package for SPSS version 27 (SPSS/IBM, Chicago). The test performed was the Multivariate analysis of variance (MANOVA), and the Post-hoc Turkey HSD's test was used to assess the possible differences in the mycotoxigenicity of fungal isolates from different provinces and seasons. Values were considered significantly different if the level of p was < 0.05.

CHAPTER FOUR

RESULTS

This chapter presents a summary of the various isolated fungal species and their attendant mycotoxins recovered from feeds and feedstuffs donated by smallholder dairy cattle farmers from Free State and Limpopo provinces, South Africa. Co-occurrences of one or more fungal species were reported, as well as effects of seasonal variation and differences in geographical locations on the toxigenicity of some of the fungal isolates were also reported in this study.

4.1 ISOLATION AND IDENTIFICATION OF FUNGI

Contamination of animal feeds and feedstuffs by fungi is a major threat to the world due to the toxins they can produce, which adversely affects the health and wellbeing of animals and humans. In this study, a total of 237 fungal isolates from 14 genera were recovered from 70 dairy cattle feeds and feed ingredients following morpho-molecular identification. Figure 4.1 indicates the macroscopic characteristics of some of the isolated fungal species on different agar plates.

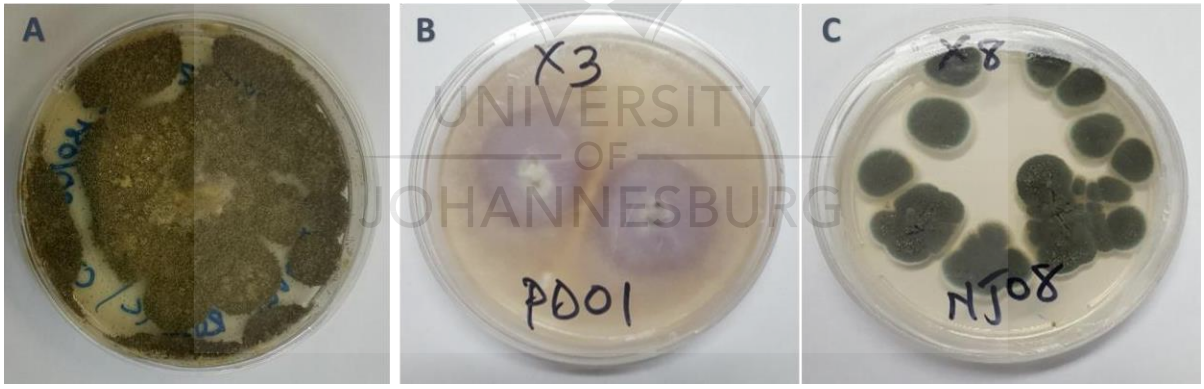


Figure 4.1: Macroscopical characteristics of isolated fungi on different agar media (A): *Aspergillus flavus* colony features on Czapek Yeast Agar (CYA) medium, (B): *Fusarium oxysporum* colony features on Potato Dextrose Agar (PDA) medium, and (C): *Penicillium crustosum* colony feature on Malt Extract Agar (MEA) medium.

Table 4.1 shows the mean fungal population represented as colony-forming units per gram of sample (CFU/g) for various dairy feeds and feed ingredients from two South African provinces (Free State and Limpopo) with raw data presented in Appendix A. Overall, the mean fungal loads (CFU/g) of the species were highly variable between the two provinces and among the feed samples, ranging from 9.3 x 10^3 to 3.6 x 10^5 CFU/g in silages and soybeans, respectively (Table 4.1). Furthermore, mean contamination levels recorded in Free State and Limpopo ranged from 9.3 x 10^3 to 3.3 x 10^5 and 2.1 x 10^4 to 3.6 x 10^5 CFU/g, respectively (Table 4.1). The highest fungal load was recorded in total mixed ration (TMR) from Free State (3.0 x 10^6 CFU/g), while the least culturable fungal population of 1.1 x 10^3 was observed in pellet from Limpopo (Appendix A). Mycological analyses also revealed that 97% (68/70) of the samples were contaminated by diverse fungi. Samples were qualified as good (count range: < 3 x 10^4 CFU/g), regular (count range: 3 x 10^4 to 7 x 10^4 CFU/g), bad (> 7x 10^4 CFU/g). Based on the mycological quality criterion, the results from this study (Appendix A) revealed that 33% (23/70) of the samples were qualified as good, 23% (16/70) as regular, and 44% (31/70) as bad.

Table 4.1: Mean fungal loads recovered from smallholder dairy cattle feeds and feedstuffs from Free State and Limpopo provinces, South Africa.

	Free State			Limpopo			
Feed	**No. of sample analysed**	**No. of positive samples**	**Mean (CFU/g)**	**Feed**	**No. of sample analysed**	**No. of positive samples**	**Mean (CFU/g)**
Grasses	2	2	1.4 x 10^5	Grasses	6	6	1.06 x 10^5
Lucerne	4	4	1.5 x 10^5	Lucerne	7	7	3.1 x 10^5
Pellet	1	1	6 x 10^4	Pellet	11	10	1.15 x 10^5
Soybean	1	1	1.1 x 10^4	Soybean	4	4	3.6 x 10^5
Silage	3	2	9 .3 x 10^3	Silage	1	1	2.1 x 10^4
TMR	22	22	3.3 x 10^5	TMR	-	-	-
Others[a]	7	7	9.5 x 10^4	Others	1	1	2.4 x 10^4

Others[a] = dairy concentrates (4), maize stover (1), molasses (2) and ramilick (1); TMR = Total Mixed Ration; CFU/g = Colony forming unit per gram of sample; No = number.

Among the 14 fungal genera recovered from the 70 dairy cattle feeds and feedstuffs, *Aspergillus*, the predominant genera, occurred at incidence rates of 44% in samples from both provinces. This was closely followed by *Fusarium* species, with incidence rates of 24 and 16% in Free State and Limpopo samples, respectively, while *Penicillium* was found in Free State and Limpopo samples at incidence rates of 11 and 16% (Table 4.2). Other fungal genera recovered in this study include *Alternaria, Cladosporium, Epicoccum, Meyerozyma, Mucor, Paecilomyces, Rhizoctonia, Rhizopus, Talaromyces, Trichoderma* and *Yeast.*

Table 4.2: Incidence rates of fungal contamination with *Aspergillus, Penicillium, Fusarium* and other fungal genera in dairy cattle feeds and feedstuffs from Free states and Limpopo provinces, South Africa.

		Isolated genera									
Feed	**Location**	***Aspergillus***	***Fusarium***	***Penicillium***	***Alternaria***	***Cladosporium***	***Epicoccum***	***Rhizopus***	***Trichoderma***	**Others**	***Yeast***
Grass	Free state	1 (17)	4 (67)	-	-	-	1 (17)	-	-	-	-
	Limpopo	8 (47)	-	3 (18)	-	-	3 (18)	1 (6)	1 (6)	1 (6)	-
Lucerne	Free state	3 (33)	4 (44)	-	1(11)	-	-	-	1 (11)	-	-
	Limpopo	11 (50)	4 (18)	2 (9)	-	-	2 (9)	1 (5)	1 (5)	1 (5)	-
Pellet	Free state	1(100)	-	-	-	-	-	-	-	-	-
	Limpopo	17 (41)	6 (15)	8 (20)	2 (5)	-	-	3 (7)	1 (2)	4 (10)	-
Soybean	Free state	2 (50)	2 (50)	-	-	-	-	-	-	-	-
	Limpopo	7 (44)	5 (31)	1 (6)	1 (6)	-	1 (6)	-	-	1 (6)	-
Silage	Free state	3 (50)	-	1 (17)	-	-	-	-	-	1 (17)	1 (17)
	Limpopo	-	1 (33)	-	-	-	-	-	-	1 (33)	1 (33)
TMR	Free state	36 (44)	16 (20)	9 (11)	3 (4)	3 (4)	2 (3)	5 (6)	3 (4)	3 (4)	1 (1)
	Limpopo	-	-	-	-	-	-	-	-	-	-
Others[a]	Free state	12 (46)	6 (23)	4 (15)	1 (4)	1 (4)	-	-	-	2 (8)	-
	Limpopo	3 (60)	1 (20)	-	-	-	-	-	1 (20)	-	-
Total	Free state	58 (44)	32 (24)	14 (11)	5 (4)	4 (3)	3 (2)	5 (4)	4 (3)	6 (3)	2 (2)
	Limpopo	46 (44)	17 (16)	14 (13)	3 (3)	-	6 (6)	5 (5)	4 (4)	8 (8)	1 (1)

Others = *Meyerozyma* (2), *Mucor* (3), *Paecilomyces* (4) *Rhizoctonia* (2) and *Talaromyces* (3); TMR = total mixed ration; Others[a] = dairy concentrates (4), maize stover (1), molasses (2) and ramilick (1); TMR = Total Mixed Ration.

As observed in Tables 4.3 and 4.4, *A. fumigatus* and *A. flavus* were the most frequent *Aspergillus* spp. recorded in the two provinces. Strains of *A. fumigatus* were detected in half (50%) of the samples from Free State and 12/30 (40%) from Limpopo, occurring most frequently in lucernes, TMR and other feeds from Free State, as well as in lucernes, soybeans and other feeds from Limpopo. Furthermore, *A. flavus* was found to occur in 38 and 47% of feeds from Free State and Limpopo, respectively. This was closely followed by *A. niger*, which occurred in 13/40 (33%) and 12/30 (40%) of feeds from Free State and Limpopo provinces. Less prominent members of the *Aspergillus* spp. recovered from the feeds are *A. candidus*, and *A. ochraceus*, found in 4 and 2 of the feed samples. Out of the 4 *A. candidus* isolates recorded in this present study, 75% was found in TMR from Free State.

The trend of *Fusarium* spp. in both provinces was observed to be different. The most dominant *Fusarium* spp. in Free State was *F. oxysporum*, occurring in 25% of the samples, followed by *F. chlamydosporum* and *F. verticillioides*, both with total frequencies of 23%. In Limpopo, *F. equiseti* was the most occurring *Fusarium* spp. recovered from 30% of the analysed samples with the highest frequency of 100% in soybeans. This was followed by *F. chlamydosporium* which occurred in 10% of feeds sourced from the province. *P. crustosum* was the only detected *Penicillium* spp. in this study, recording incidence rates of 35 and 47% in samples from Free State and Limpopo, respectively, with the highest frequency found in pellets (80%) from Limpopo. It is worth noting that very high co-occurrence of two or more fungal species, particularly, *A. fumigatus*, *A. flavus* and *A. niger* were noticed in most of the feeds, including pellets, grasses, lucernes and soybeans, particularly from Limpopo

Table 4.3: Absolute and relative % frequencies of *Aspergillus, Fusarium and Penicillium* species distributed in dairy cattle feeds and feed ingredients from Free State, South Africa.

	Contaminated samples							
Fungal species	**Grasses/hay (2)**	**Lucerne (4)**	**Pellet (1)**	**Silage (3)**	**Soybean (1)**	**TMR (22)**	**Others (7)**	**Total (40)**
***Aspergillus* species**								
A. flavus	-	-	1 (100)	2 (67)	1(100)	8 (36)	3 (43)	15 (38)
A. fumigatus	1 (50)	2 (50)	-	1 (33)	-	12 (55)	4 (57)	20 (50)
A. niger	-	-	-	-	1(100)	9 (41)	3 (43)	13 (33)
A. ochraceus	-	-	-	-	-	1(5)	1 (14)	2 (5)
A. terreus	-	1 (25)	-	-	-	3(14)	1(14)	5 (13)
A. candidus	-	-	-	-	-	3(14)	-	3 (8)
***Fusarium* species**								
F. brachygibbosum	-	-	-	-	-	-	1 (14)	1 (3)
F. chlamydosporum	1 (50)	-		-	-	5 (23)	3 (43)	9 (23)
F. equiseti	-	1 (25)		-	-	1 (5)	-	2 (5)
F. incarnatum	-	-	-	-	-	1 (5)	-	1 (3)
F. oxysporum	1 (50)	2 (50)	-	-	1(100)	5 (23)	1 (14)	10 (25)
F. verticillioides	2 (100)	1 (25)	-	-	1(100)	4 (18)	1 (14)	9 (23)
***Penicillium* species**								
P. crustosum	-	-	-	1 (33)	-	9 (41)	4 (57)	14 (35)
Total	**5**	**7**	**1**	**4**	**4**	**61**	**22**	**104**

Others[a] = dairy concentrates (4), maize stover (1), molasses (2) and ramilick (1); TMR = Total Mixed Ration.

Table 4.4: Absolute and relative % frequencies of Aspergillus, *Fusarium* and *Penicillium* species distributed in dairy cattle feeds and feed ingredients from Limpopo, South Africa.

	Contaminated samples							
Fungal species	**Grasses/hay (6)**	**Lucerne (7)**	**Pellet (10)**	**Silage (1)**	**Soybean (4)**	**TMR (0)**	**Others (1)**	**Total (30)**
***Aspergillus* species**								
A. flavus	4 (67)	3 (43)	5 (50)	-	1 (25)	-	1 (100)	14 (47)
A. fumigatus	2 (33)	4 (57)	3 (30)	-	2 (50)	-	1 (100)	12 (40)
A. niger	2 (33)	3 (43)	4 (40)	-	2 (50)	-	1 (100)	12 (40)
A. ochraceous	-	-	-	-	-	-	-	
A. terreus	-	1 (14)	4 (40)	-	2 (50)	-	-	7 (23)
A. candidus	-	-	1 (10)	-	-	-	-	1 (3)
***Fusarium* species**								
F. brachygibbosum	-	-	-	1 (100)	-	-	1 (100)	2 (7)
F. chlamydosporum		1 (14)	1 (10)	-	1 (25)	-	-	3 (10)
F. equiseti	-	3 (43)	2 (20)	-	4 (100)	-	-	9 (30)
F. incarnatum	-	-	1 (10)	-	-	-	-	1 (3)
F. oxysporum	-	-	2 (20)	-	-	-	-	2 (7)
F. verticillioides	-	-	-	-	-	-	-	-
***Penicillium* species**								
P. crustosum	3 (50)	2 (28)	8 (80)	-	1 (25)	-	-	14 (47)
Total	**11**	**17**	**31**	**1**	**13**		**4**	**77**

Others[a] = dairy concentrates (4), maize stover (1), molasses (2) and ramilick (1); TMR = Total Mixed Ration.

Based on the phylogenetic analysis, the sequences were grouped into 13 clades (Figure 4.2 and 4.3). SH3001 was grouped in clade 1 with confirmed *A. candidus* (KY2602665 and MH865265). SH5001 was grouped together with *A. terreus* (MN326736) in clade 2. SH8001 was associated with *A. niger* isolates in clade 3. SH1201 was also found in clade 4 with *A. ochraceus* (MH270530) with 86% bootstrap value, while SH9001 was grouped in the same clade as *A. flavus* (MG659646). The isolate SH1001 was associated with *A. fumigatus* isolates in clade 6. In a similar analysis, SH1101 was classified in the same clade as *F. brachygibbosum* (KP881513) in a phylogenetic tree for *Fusarium* and *Penicillium* spp., as shown in the Figure 4.3. Also, isolate SH2701 was grouped in clade 8 with *F. chlamydosporum* (MW931873), while isolate SH1301 was grouped with two or more confirmed *F. oxysporum* isolates (MG407705 and MW739949) in clade 9. Furthermore, isolate SH2001 was grouped with confirmed *F. verticillioides* isolate (MN871541) in clade 10 with 83% bootstrap value. SH4001 was found in a clade that included *F. equiseti (*MT626672), whereas SH2011 was grouped with *F. incarnatum* isolates in clade 12. Finally, SH6001 and SH6004 were grouped along with a confirmed *P. crustosum* isolate (MH270547) in clade 13.

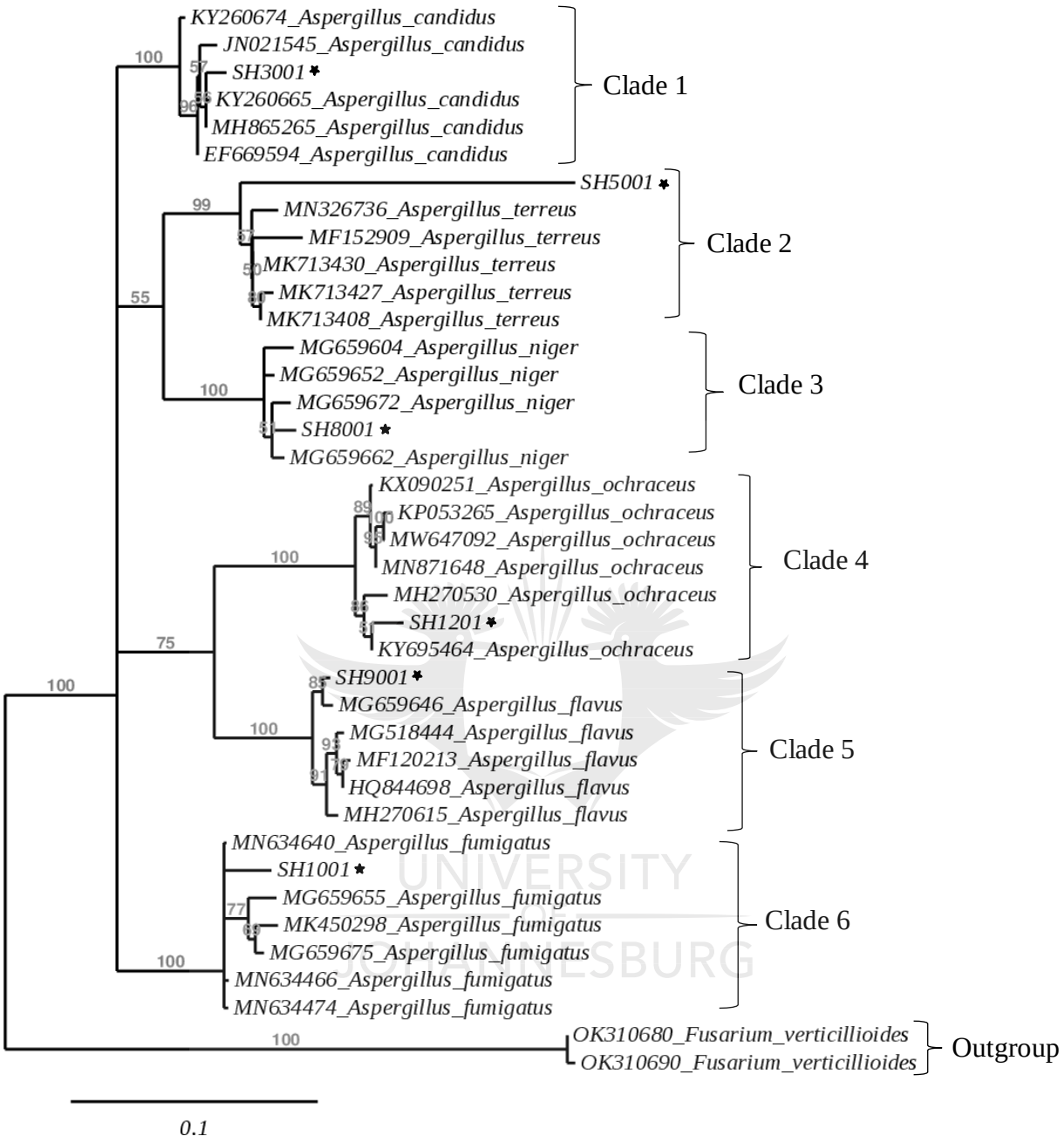


Figure 4.2: Neighbour-joining phylogenetic tree showing the phylogenetic relationship within the genus *Aspergillus* isolates from dairy cattle feeds based on the sequences of the ITS region. Bootstraps percentage of the Neighbour joining are presented at the nodes, while the number of substitutions of nucleotide sequences per site is shown on the scale bar below trees. The phylogram is rooted (outgroup) with *Fusarium verticillioides*.

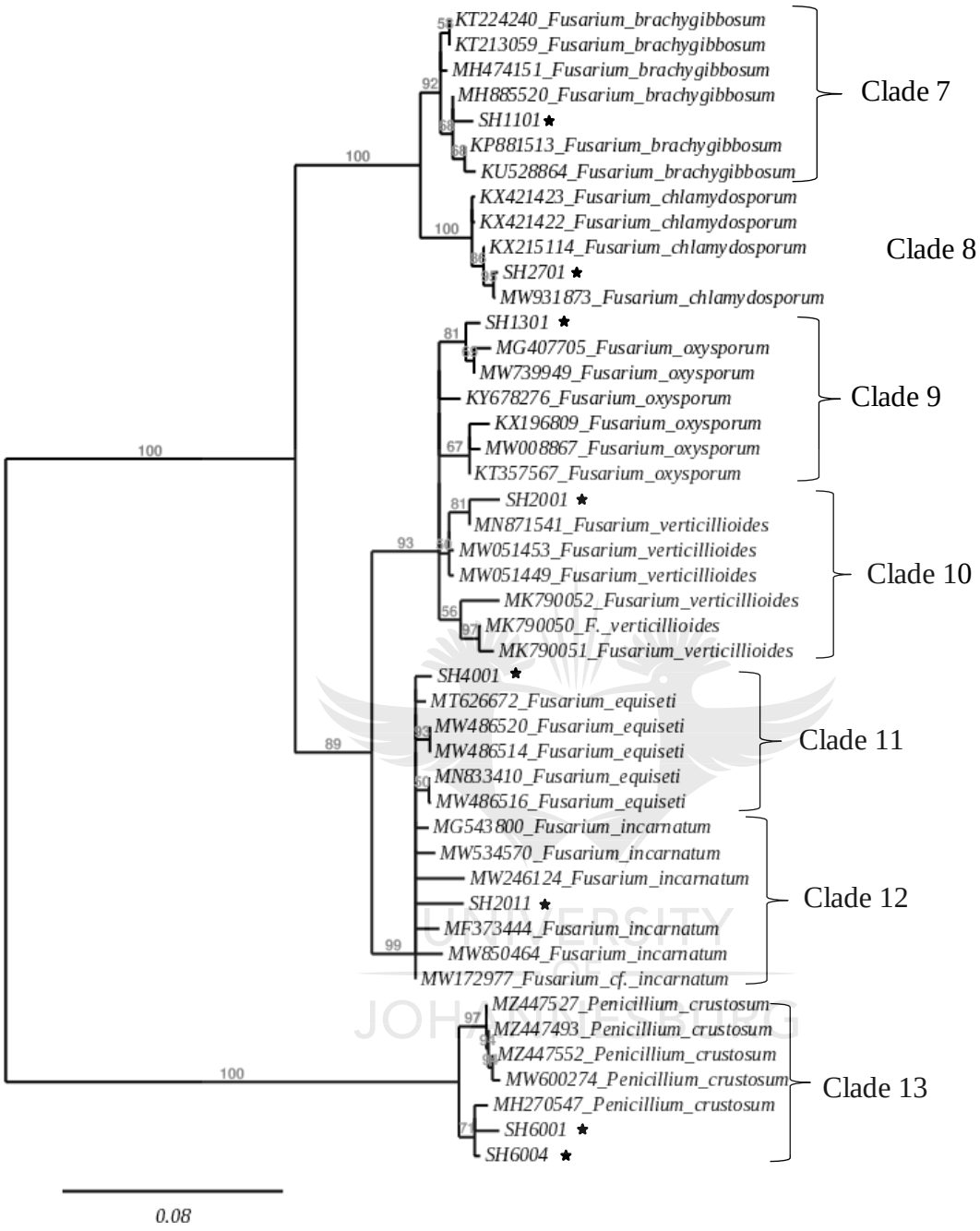


Figure 4.3: Neighbour-joining phylogenetic tree showing the phylogenetic relationship of *Fusarium* and *Penicillium* isolates recovered from dairy cattle feeds based on the sequences of the ITS region. Bootstraps percentage of the Neighbour joining are presented at the nodes, while number of substitutions of nucleotide sequences per site is shown on the scale bar below trees.

The ITS-based identification of some *Aspergillus*, *Fusarium* and *Penicillium* species isolated from dairy feeds and feedstuffs in relation to Gen Bank are shown in Tables 4.5 and 4.6.

Table 4.5: ITS-based identification of some *Aspergillus* species recovered from dairy feeds and feedstuffs in relation to Gen Bank.

Species Name	Accession No	Geographical Region	Reference
Africa clade			
A. candidus	JNO21545	South Africa	Mouton *et al*., 2011
	SH3001	South Africa	
A. flavus	MG659646	Zimbabwe	Nleya *et al.*, 2017
A. flavus	MH270615	Zimbabwe	Nleya *et al.*, 2017
A. flavus	MG518444	Nigeria	Adetunji and Mwanza, 2017
	SH9001	South Africa	
A. fumigatus	MG659655	Zimbabwe	Nleya *et al*., 2017
A. fumigatus	MG659675	Zimbabwe	Nleya *et al*., 2017
A. fumigatus	MN634474	South Africa	Selvarajan *et al.*, 2019
A. fumigatus	MN634466	South Africa	Selvarajan *et al.*, 2019
A. fumigatus	MN634640	South Africa	Selvarajan *et al.*, 2019
	SH1001	South Africa	
A. niger	MG659652	Zimbabwe	Nleya *et al*., 2017
A. niger	MG659662	Zimbabwe	Nleya *et al*., 2017
A. niger	MG659672	Zimbabwe	Nleya *et al*., 2017
A. niger	MG659604	Zimbabwe	Nleya *et al*., 2017
	SH8001	South Africa	
A. ochraceus	KP053265	Egypt	Ammar, 2014
A. ochraceus	MW647092	Egypt	Moharram, *et al*., 2019
A. ochraceus	MH270530	Zimbabwe	Nleya, 2017
	SH1201	South Africa	
A. terreus	MK713427	Ghana	Frimpong, 2019
A. terreus	MK713408	Ghana	Frimpong, 2019
A. terreus	MK713430	Ghana	Frimpong, 2019
	SH5001	South Africa	
Asia clade			
A. candidus	KY260674	India	Kumari and Ghosh, 2016
A. candidus	KY260665	India	Kumari and Ghosh, 2016
A. fumigatus	MK450298	China	Liu and Qin, 2019
A. ochraceus	KX090251	China	Yu and Zhou, 2016
A. ochraceus	KY695464	Iran	Kasfi *et al*., 2017

Species Name	Accession No	Geographical Region	Reference
A. terreus	MN326736	India	Aruna, *et al*., 2019
A. terreus	MF152909	India	Prameeladevi *et al*, 2017
America clade			
A. candidus	EF669594	USA	Peterson, 2008
Europe clade			
A. flavus	HQ844698	Italy	Accinelli *et al*., 2012
A. candidus	MH865265	Netherland	Vu *et al*., 2017
Outgroup			
F. verticillioides	OK310680	Iraq	Almatakeez and Bluhm, 2021
F. verticillioides	OK310690	Iraq	Almatakeez and Bluhm, 2021

Table 4.6: ITS-based identification of some *Fusarium* and *Penicillium* species recovered from dairy cattle feeds and feedstuffs in relation to Gen Bank.

Species Name	Accession No.	Geographical location	Reference
Africa clade			
F. brachygibbosum	KU528864	Tunisia	Rahma, 2016
	SH1101	South Africa	
F. chlamydosporum	MW931873	Kenya	Karani *et al*., 2021
F. chlamydosporum	KX215114	South Africa	Adekoya *et al*., 2016
	SH2701	South Africa	
F. chlamydosporum	MN882831	Nigeria	Ezekiel *et al*., 2019
F. equiseti	MW486516	Uganda	Wokorach *et al*., 2021
F. equiseti	MW486514	Uganda	Wokorach *et al*., 2021
F. equiseti	MW486520	Uganda	Wokorach *et al*., 2021
	SH4001	South Africa	
F. incarnatum	MF373444	Egypt	Khattab and Ziedan, 2017
	SH2011	South Africa	
F. oxysporum	MW008867	Tunisia	Rahma, 2016
F. oxysporum	KT357567	Kenya	Karani *et al*., 2021
	SH1301	South Africa	
F. verticillioides	MW051449	Egypt	Gomaa, 2020
F. verticillioides	MW051453	Egypt	Gomaa, 2020
	SH2001	South Africa	
P. crustosum	MH270547	Zimbabwe	Nleya *et al*., 2018
	SH6001	South Africa	
	SH6004	South Africa	
Asia clade			
F. brachygibbosum	KP881513	India	Sharma *et al*., 2015
F. brachygibbosum	MH885520	India	Shirasangi *et al*., 2018
F. brachygibbosum	KT224240	China	Wang and Wu, 2015
F. equiseti	MT626672	China	Dong, 2020
F. incarnatum	MG543800	India	Thirumalaisamy, 2019
F. incarnatum	MW534570	India	Li and Yang, 2021
F. incarnatum	MW850464	India	Parihar *et al*., 2021
F. incarnatum	MW172977	China	Yang *et al*., 2021
F. oxysporum	MG407705	China	Bao, 2017
F. oxysporum	KY678276	India	Dubey *et al*., 2017
F. oxysporum	MW739949	India	Mahadevakumar *et al*., 2021
F. oxysporum	KX196809	China	Yu and Saravanakumar, 2016
F. verticilliodes	MK790050	India	K, A *et al*, 2019
F. verticilliodes	MK790051	India	K, A *et al*, 2019
F. verticilliodes	MK790052	India	K, A *et al*, 2019

Species Name	Accession No.	Geographical location	Reference
F. verticillioides	MN871541	China	Li *et al*., 2019
America clade			
F. brachygibbosum	MH474151	USA	Ndinga Muniania, 2018
F. chlamydosporum	KX421422	Brazil	Poltronieri *et al*., 2016
F. chlamydosporum	KX421423	Brazil	Poltronieri *et al*, 2016
Europe clade			
F. equiseti	MN833410	Switzerland	Haenzi *et al*., 2019
P. crustosum	MZ447552	Poland	Mikolajczak *et al*., 2021
P. crustosum	MZ447493	Poland	Mikolajczak *et al*., 2021
P. crustosum	MZ447527	Poland	Mikolajczak *et al*., 2021

4.2 MYCOTOXIGENIC POTENTIALS OF *ASPERGILLUS, FUSARIUM* AND *PENICILLIUM* ISOLATES ISOLATED FROM DAIRY CATTLE FEEDS

The presence of AFs, OTA, DON, and ZEN in the tested samples was confirmed using a semi-quantitative thin-layer chromatography (TLC) technique. To aid in the idYTPentification of the attendant mycotoxins, the retardation factors (RF_1 and RF_2) and colours of the individual spot on TLC plates were determined, marked, and compared with those of standard mycotoxins. The fluorescences of AFB_1, AFB_2 and ZEN viewed under ultraviolet light showed that some isolates were positive, showing a light blue for AFB_1, AFB_2 and ZEN as indicated in Figure 4.4. It is important to mention that FB_1 was suspected in this study but could not be confirmed due to lack of reference standard. Among the 104 *Aspergillus* isolates recovered from the feeds and feedstuffs, *A. flavus* was the only AFs producer, producing only aflatoxin B types (AFB_1 and AFB_2). AFG_1 and AFG_2 were not detected in this study due to the absence of some aflatoxigenic strains that produce aflatoxin G types, such as *A. parasiticus* in the samples. Among the 29 *A. flavus* isolated in this study, 24 (82%) and 10 (35%) produced AFB_1 and AFB_2, while 10 (35%) produced both toxins. It is important to mention that none of the *A. flavus* strains isolated from silage produces AFB_2. Furthermore, 12/15 (80%) and 12/14 (86%) of *A. flavus* strains isolated from Free State and Limpopo were tested positive for AFB_1 and AFB2, respectively. It is also important to mention that of the 24 aflatoxigenic strains of *A. flavus* isolated in this study, 75 and 25% of them were from feeds sourced during summer and winter, respectively (Appendix B, Table 1).

While all the strains of *A. flavus* isolated from pellets, lucernes and other samples were aflatoxigenic, 50% of those from soybeans and silages, and 75% of those from TMR and grasses produced similar mycotoxins. None of the *A. niger,* A. *terreus, A. candidus, A. fumigatus, and A. ochraceus* strains isolated in this study produced any of the aflatoxins tested for. In addition, no strain of *A. niger* and *A. ochracheus,* the notable OTA producers, produced the mycotoxin. ZEN (the only *Fusarium* toxin detected in this study) was produced by *F. equiseti* and *F. oxysporum* at incidence rates of 50% (5/10) and 58% (7/12), respectively. None of the strains of *F. verticillioides, F. chlamydosporium, F. brachygibbosum* and *F. incarnatum* produce any of the mycotoxins tested for. It must be emphasised that 58 and 42% of the ZEN produced by the *Fusarium* isolates in this study were isolated from feeds sourced during summer and winter, respectively. Lastly, none of the *Fusarium* isolates recovered in this study produced DON.

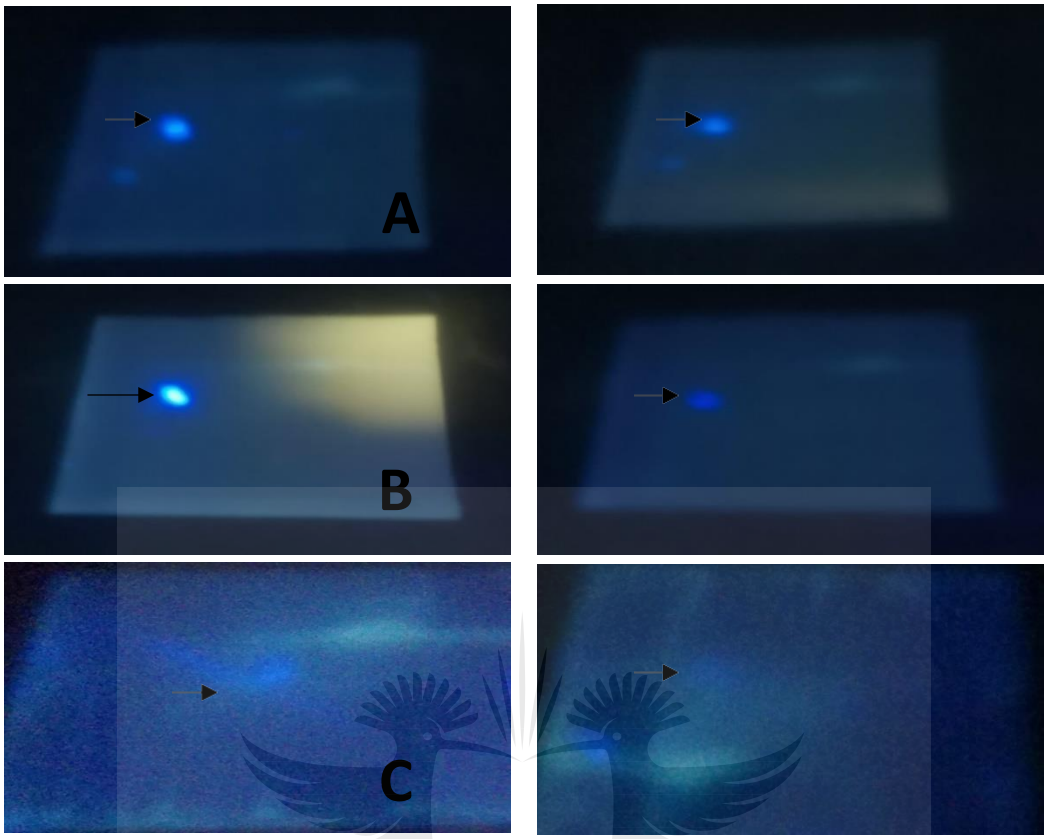


Figure 4.4 View of silica gel coated two-dimensional aluminium baked TLC plates for A: AFB_1 standard (*left*) and AFB_1 produced by *Aspergillus flavus* isolated from grasses (*right*), B: AFB_2 standard (*left*) and AFB_2 produced by *Aspergillus flavus* isolated from pellet (*right*), C: zearalenone standard (*left*) and zearalenone produced by *Fusarium oxysporum* isolated from TMR (*right*).

4.3 QUANTIFICATION OF MYCOTOXINS BY LIQUID CHROMATOGRAPHY WITH TANDEM MASS SPECTROMETRY (LC-MS/MS).

Mycotoxins produced by *Aspergillus, Penicillium* and *Fusarium* isolates recovered in this study were further quantified using LC-MSLMS. Method validation was performed in terms of retention time, linearity, limit of detections (LODs), limit of quantifications (LOQs), and recovery. The retention times for all the analysed mycotoxins varied from 4.90 to 9.30 mins (Table 4.7). The analytical method showed adequate linearity with R^2 for all the levels of mycotoxins ranging from

0.9966 to 0.9995 (Table 4.7). The LODs and LOQs of different analytes ranged from 0.01 to 4.42 and 0.04 to 13.40 µg/kg, respectively, while the apparent mean recoveries for all the tested mycotoxins ranged from 71.4 to 101.9 (Table 4.7), within the acceptable range of required performance criteria. (EC, 2006).

Table 4.7: The matrix-matched calibration curve parameters, LOD, LOQ and recovery values for CYA medium.

Mycotoxin	Calibration points	Ret. Time (min)	R^2	Slope	LOD (µg/kg)	LOQ (µg/kg)	Recovery
AFB_1	0.5, 1, 50, 250	7.84	0.9986	657.99	0.04	0.14	80.9
AFB_2	0.5, 1, 100, 250	7.64	0.9988	965.93	0.02	0.07	101.9
AFG_1	1, 10, 50, 500	7.45	0.9995	748.77	0.06	0.19	90.3
AFG_2	1, 10, 25, 250	7.25	0.9994	420.65	0.05	0.17	93.3
OTA	25, 50, 250, 500	9.30	0.9987	1864.73	0.01	0.04	98.3
DON	1, 10, 100, 250	4.90	0.9970	5.45	4.42	13.40	71.4
ZEN	0.5, 25, 50, 250	7.75	0.9966	11.09	0.74	2.24	92.9

Aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZEN); Ret = Retention; R^2 = Coefficient of determination; LOD = limit of detection; LOQ = limit of quantification.

Figures 4.5 and 4.6 present MRM chromatograms showing the production of aflatoxins (AFB_1 and AFB_2) by *A. flavus* isolate and ZEN by *F. oxysporum* isolate, and calibration curves of mycotoxin standards on LC-MS/MS.

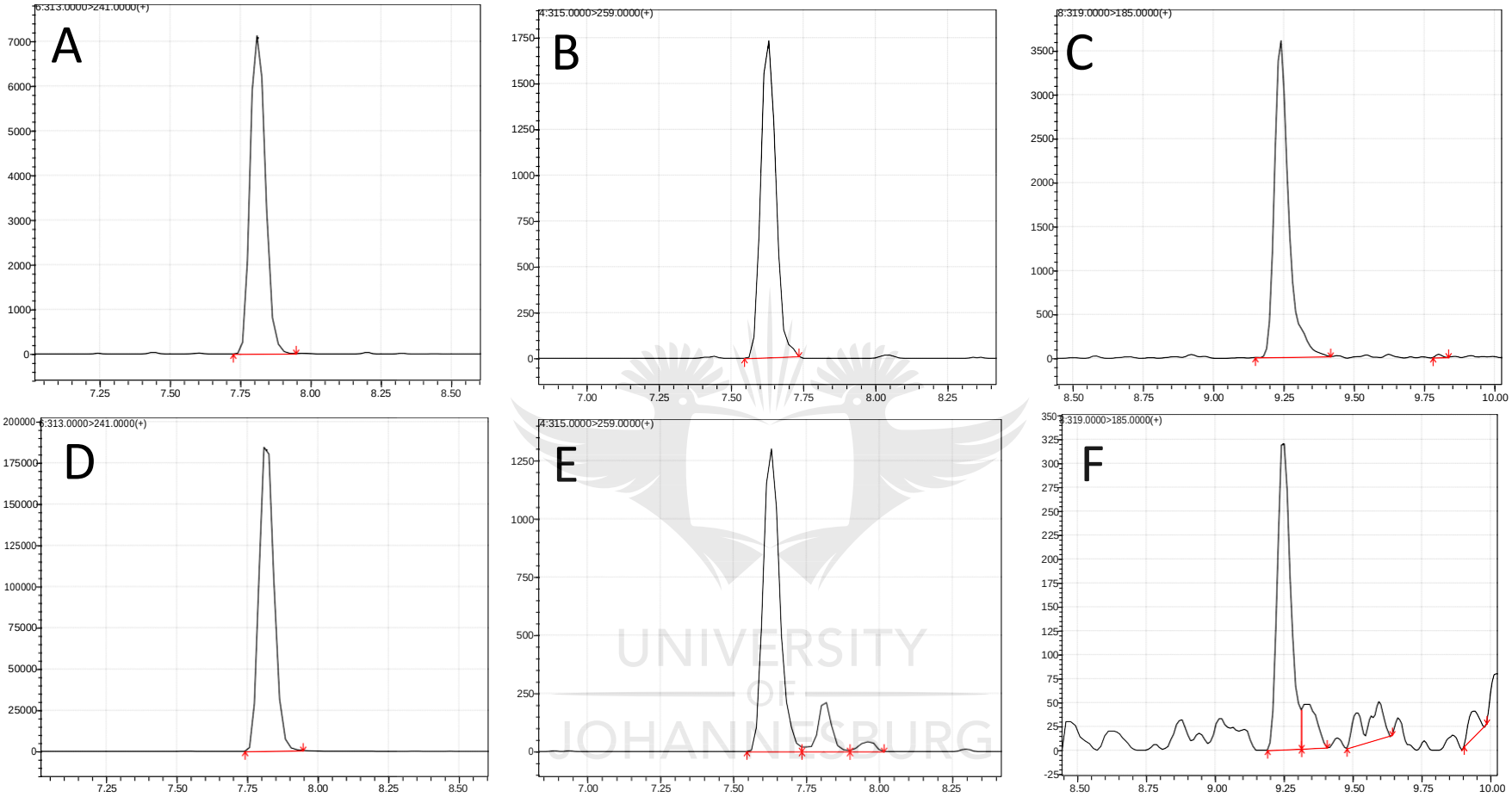
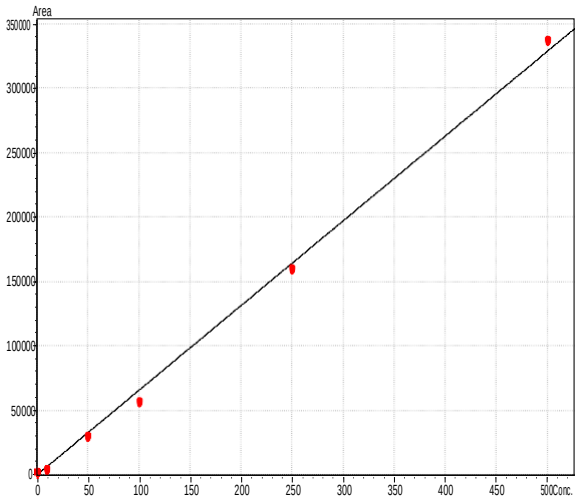
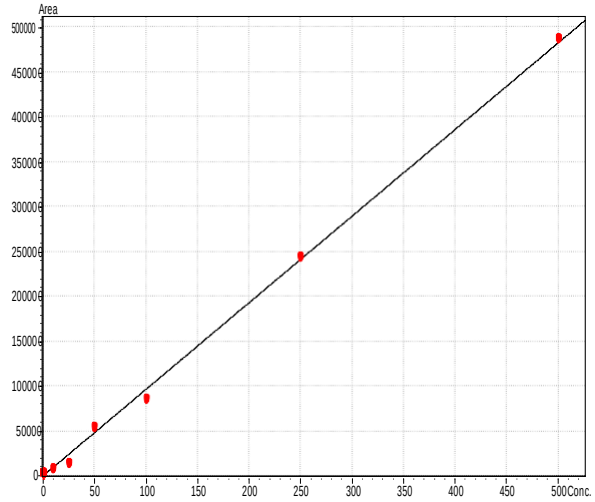


Figure 4.5: Chromatograms of mycotoxins. A = aflatoxin B_1 standard, B = aflatoxin B_2 standard, C = zearalenone standard, while D and E = aflatoxin B_1 and aflatoxin B_2 produced by *Aspergillus flavus* isolated from pellet and F = zearalenone produced by *Fusarium oxysporum* isolated from TMR.

(A)



(B)



(C)

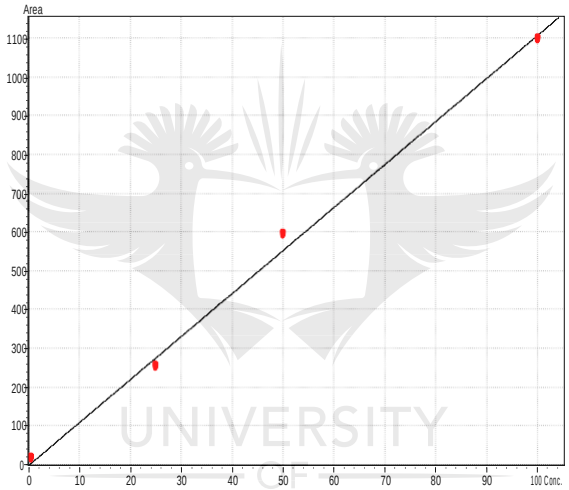


Figure 4.6: Calibration curve of mycotoxin standards on LC-MS/MS. A = AFB_1, B = AFB_2 and C = ZEN.

Results of the range and mean concentrations of the mycotoxins produced by *Aspergillus*, *Penicillium* and *Fusarium* isolates recovered from dairy cattle feeds and feed ingredients are summarised in Table 4.8.

As found in this study, AFB_1 production by *A. Flavus* was recorded with mean concentrations of 101.97, 1.94, 220.51, 0.69, 106.59, 0.8, and 8.31 µg/kg in grasses, lucernes, pellets, silage, TMR, soybeans and other feed samples accordingly (Table 4.8). The maximum AFB_1

concentration (1045.8 µg/kg) was recovered in pellet sample from Limpopo, while the minimum concentration (0.22 µg/kg) was found in TRM from Free State (Appendix B, Table 1). Regarding AFB_2, mean concentrations of 0.89, 0.21, 2.08, 1.27, 0.11 and 0.78 µg/kg were recorded in grasses, lucernes, pellets, TMR, soybeans and other feeds, respectively (Table 4.8). The highest concentration (3.44 µg/kg) of AFB_2 was observed in pellet from Limpopo, while minimum concentration (0.11 µg/kg) was found in soybean and TMR from Free State (Appendix B, Table 1).

Among the *Fusarium* toxins, ZEN was the only one detected in this study, produced by *F. equseti and F. oxysporum*. The highest concentration of ZEN (97.18 µg/kg) was produced by *F. equiseti* recovered from Free State sample (pellet), while the least concentration of 5.20 µg/kg was produced by *F. oxysporum* isolated from Limpopo TMR (Appendix B, Table 2). Also, *P. crustosum*, the only *Penicillium* spp. recovered in this current study produced no detectable mycotoxin (Table 4.8).

4.8: Mycotoxins production by *Aspergillus, Penicillium* and *Fusarium* species isolated from dairy cattle feeds and feedstuffs in South Africa.

Fungal source	Isolated species	No. of strain isolated[a]	Toxin Produced	Ranged of toxin produced (µk/kg)	Mean
Grasses/hay	***Aspergillus* species (9)**				
	A. flavus	4 (3,1)	AFB_1	2.36 - 298.92	101.97
			AFB_2	0.89	0.89
	A. fumigatus	3 (0)	ND	ND	ND
	A. niger	2 (0)	ND	ND	ND
	A. ochracheus	ND	ND	ND	ND
	A. terreus	ND	ND	ND	ND
	A. candidus	ND	ND	ND	ND
	***Fusarium* species (4)**				
	F. brachygibossum	ND	ND	ND	ND
	F. chlamydosporum	1 (0)	ND	ND	ND
	F. equiseti	ND	ND	ND	ND
	F. incarnatum	ND	ND	ND	ND
	F. oxysporum	1 (1)	ZEN	16.29	16.29
	F. verticillioides	2 (0)	ND	ND	ND
	Penicillium* species *(3)				
	P. crustosum	3 (0)	ND	ND	ND
Lucerne	***Aspergillus* species (14)**				
	A. flavus	3 (3,1)	AFB_1	0.93 - 2.95	2.01
			AFB_2	0.21	0.21
	A. fumigatus	6 (0)	ND	ND	ND
	A. niger	3 (0)	ND	ND	ND
	A. ochraceus	ND	ND	ND	ND
	A. terreus	2 (0)	ND	ND	ND
	A. candidus	ND	ND	ND	ND
	***Fusarium* species (8)**				
	F. brachygibossum	ND	ND	ND	ND
	F. chlamydosporum	1 (0)	ND	ND	ND
	F. equiseti	4 (2)	ZEN	7.64 - 9.08	8.36
	F. incarnatum	ND	ND	ND	ND
	F. oxysporum	2 (1)	ZEN	15.90	15.90
	F. verticillioides	1 (0)	ND	ND	ND
	Penicillium* species (2*)				
	P. crustosum	2 (0)	ND	ND	ND
Pellet	***Aspergillus* species (18*)***				
	A. flavus	6 (6,4)	AFB_1	0.43 - 1045.8	220.51
			AFB_2	0.13 - 3.44	2.08
	A. fumigatus	3 (0)	ND	ND	ND
	A. niger	4 (0)	ND	ND	ND
	A. ochraceus	ND	ND	ND	ND
	A. terreus	4 (0)	ND	ND	ND
	A. candidus	1 (0)	ND	ND	ND

Fungal source	Isolated species	No. of strain isolated[a]	Toxin Produced	Ranged of toxin produced (µk/kg)	Mean
	***Fusarium* species (6)**				
	F. brachygibossum	ND	ND	ND	ND
	F. chlamydosporum	1 (0)	ND	ND	ND
	F. incarnatum	1 (0)	ND	ND	ND
	F. equiseti	2 (2)	ZEN	19.06 - 97.18	58.12
	F. oxysporum	2 (1)	ZEN	7.80	7.80
	F. verticillioides	ND	ND	ND	ND
	***Penicillium* species (8)**				
	P. crustosum	2 (8)	ND	ND	ND
Silage	***Aspergillus* species (3)**				
	A. flavus	2 (1)	AFB_1	0.69	0.69
	A. fumigatus	1 (0)	ND	ND	ND
	A. niger	ND	ND	ND	ND
	A. ochraceus	ND	ND	ND	ND
	A. terreus	ND	ND	ND	ND
	A. candidus	ND	ND	ND	ND
	***Fusarium* species (1)**				
	F. brachygibossum	1 (0)	ND	ND	ND
	F. chlamydosporum	ND	ND	ND	ND
	F. equiseti	ND	ND	ND	ND
	F. incarnatum	ND	ND	ND	ND
	F. oxysporum	ND	ND	ND	ND
	F. Verticillioides	ND	ND	ND	ND
	***Penicillium* species (1)**				
	P. crustosum	1 (0)	ND	ND	ND
TMR	***Aspergillus* species (36)**				
	A. flavus	8 (6,2)	AFB_1	0.22 - 576.14	106.59
			AFB_2	0.11 - 2.42	1.27
	A. fumigatus	12 (0)	ND	ND	ND
	A. niger	9 (0)	ND	ND	ND
	A. ochraceus	1 (0)	ND	ND	ND
	A. terreus	3 (0)	ND	ND	ND
	A. candidus	3 (0)	ND	ND	ND
	***Fusarium* species (16)**				
	F. brachygibossum	ND	ND	ND	ND
	F. chlamydosporum	5 (0)	ND	ND	ND
	F. equiseti	1 (1)	ZEN	8.69	8.69
	F. incarnatum	1 (0)	ND	ND	ND
	F. oxysporum	5 (3)	ZEN	5.20 - 11.09	8.01
	F. verticillioides	4 (0)	ND	ND	ND

Fungal source	Isolated species	No. of strain isolated[a]	Toxin Produced	Ranged of toxin produced (µk/kg)	Mean
	***Penicillium* species (9)**				
	P. crustosum	9 (0)	ND	ND	ND
Soybean	***Aspergillus* (9)**				
	A. flavus	2 (1,1)	AFB_1	0.8	0.8
			AFB_2	0.11	0.11
	A. fumigatus	2 (0)	ND	ND	ND
	A. ochraceus	ND	ND	ND	ND
	A. niger	3 (0)	ND	ND	ND
	A. terreus	2 (0)	ND	ND	ND
	A. candidus	ND	ND	ND	ND
	***Fusarium* species (7)**				
	F. brachygibossum	ND	ND	ND	ND
	F. chlamydosporum	1 (0)	ND	ND	ND
	F. Equiseti	4 (0)	ND	ND	ND
	F. incarnatum	ND	ND	ND	ND
	F. oxysporum	1 (0)	ND	ND	ND
	F. verticillioides	1 (0)	ND	ND	ND
	***Penicillium* species (1)**				
	P. crustosum	1 (0)	ND	ND	ND
Others[a]	***Aspergillum* species (15)**				
	A. flavus	4 (4,1)	AFB_1	0.38 - 18.85	8.31
			AFB_2	0.75	0.75
	A. fumigatus	5 (0)	ND	ND	ND
	A. niger	4 (0)	ND	ND	ND
	A. ochraceus	1 (0)	ND	ND	ND
	A. terreus	1 (0)	ND	ND	ND
	A. candidus	ND	ND	ND	ND
	***Fusarium* species (7)**				
	F. brachygibossum	2 (0)	ND	ND	ND
	F. chlamydosporum	3 (0)	ND	ND	ND
	F. equiseti	ND	ND	ND	ND
	F. incarnatum	ND	ND	ND	ND
	F. oxysporum	1 (1)	ZEN	12.52	12.52
	F. verticillioides	1 (0)	ND	ND	ND
	***Penicillium* species (4)**				
	P. crustosum	4 (0)	ND	ND	ND

AFB_1 = aflatoxin B_1; AFB_2 = aflatoxin B_2; a = number of positive isolates; ND = not detected; others[a] = dairy concentrates, molasses, ramilick, TMR = Total Mixed Ration.

4.4 INTERACTIVE EFFECTS OF SEASON AND PROVINCE ON AFB_1 AND AFB_2 PRODUCTION BY *A. flavus* AND ZEN BY THE *FUSARIUM* ISOLATES (*F. equiseti* and *F. oxysporum).*

Table 4.9 shows the multivariate analysis of variance (MANOVA) for AFB_1 production by *A. flavus* in relation to season and province and their interaction. This revealed that all the single (province and season) and two-factor (province X season) had no significant effect ($P > 0.05$) on AFB_1 production. Among the three factors tested, season had the greatest effect (VR = 3.006). It was also observed that minimum mean concentration (1.9 µk/kg) of AFB_1 was produced by isolates recovered from Limpopo winter samples, while the maximum mean concentration of 162.07 µk/kg was detected in isolates from Limpopo summer samples (Figure 4.7).

Table 4.9: MANOVA for aflatoxin B_1 (AFB_1) production (µg/kg) by *Aspergillus flavus* in dairy cattle feeds and feedstuffs in relation to season, province, and their interaction.

Effect	DF	MS	VR	P
Province	1	3641.793	0.066	0.799
Season	1	167004.885	3.006	0.087
Province X season	1	47307.755	0.852	0.359
Error	68	55556.066		
Total	72			

DF = degree of freedom; MS = mean square; VR = variance ratio; P = probability at $P \leq 0.05$.

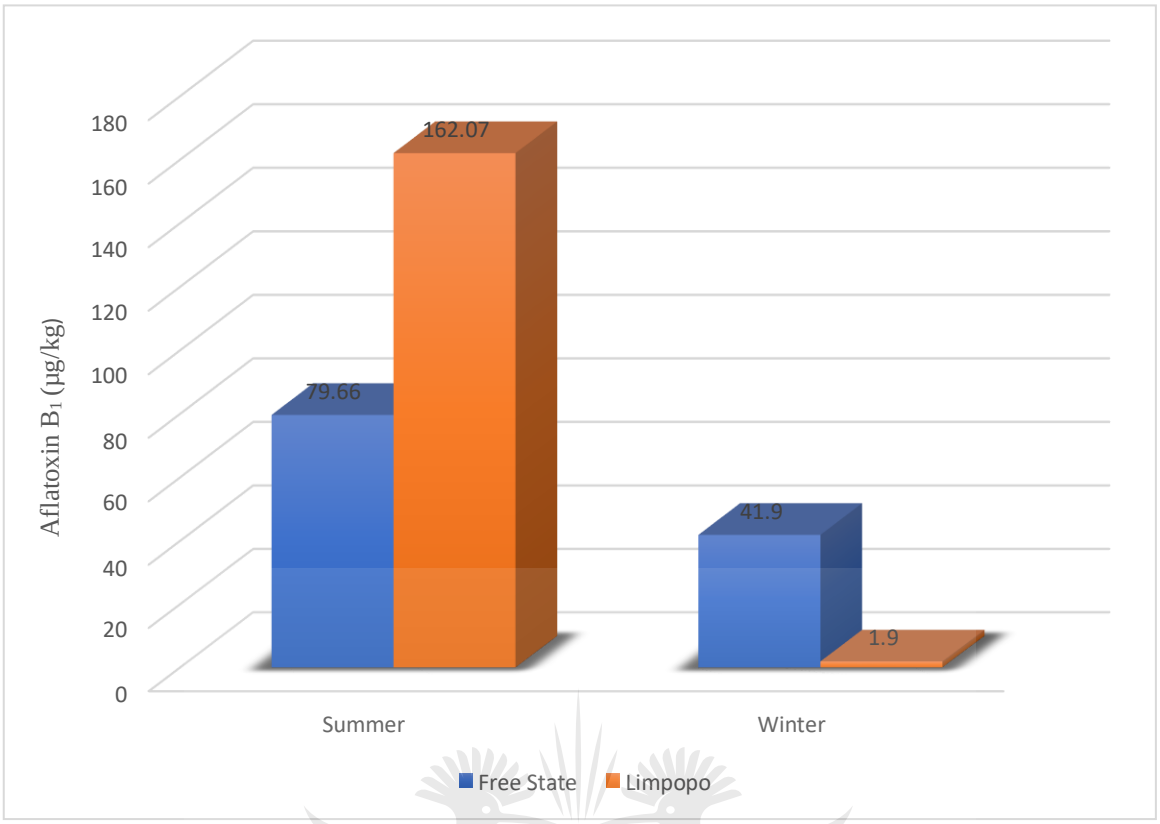


Figure 4.7: Mean concentrations of aflatoxin B_1 (AFB_1) produced by *Aspergillus flavus* isolated from dairy cattle feeds and feedstuffs from Free State and Limpopo provinces, South Africa.

The MANOVA for AFB_2 produced by *A. flavus* (Table 4.10) showed that all the single factors had no significant effect ($P > 0.05$), while the double factor (province X season) had a significant effect ($P < 0.05$) on the mycotoxin production capacity of the fungi. This was also supported by the high variance ratio value (11.750) recorded by the interactive effect (province X season). Furthermore, Figure 4.7 indicated that AFB_2 mean concentration was lower in Limpopo winter samples (0.21 µk/kg), while maximum mean concentration (2.82 µk/kg) was produced by *A. flavus* recovered from Free State winter samples.

Table 4.10: MANOVA for aflatoxin B_2 (AFB_2) production (µg/kg) by *Aspergillus flavus* in dairy cattle feeds and feedstuffs in relation to season, province, and their interaction.

Effect	DF	MS	VR	P
Province	1	4.042	3.540	0.71
Season	1	0.187	0.163	0.689
Province X season	1	13.415	11.750	0.002
Error	26	1.142		
Total	30			

DF = degree of freedom; MS = mean square; VR = variance ratio; P = probability at $P \leq 0.05$.

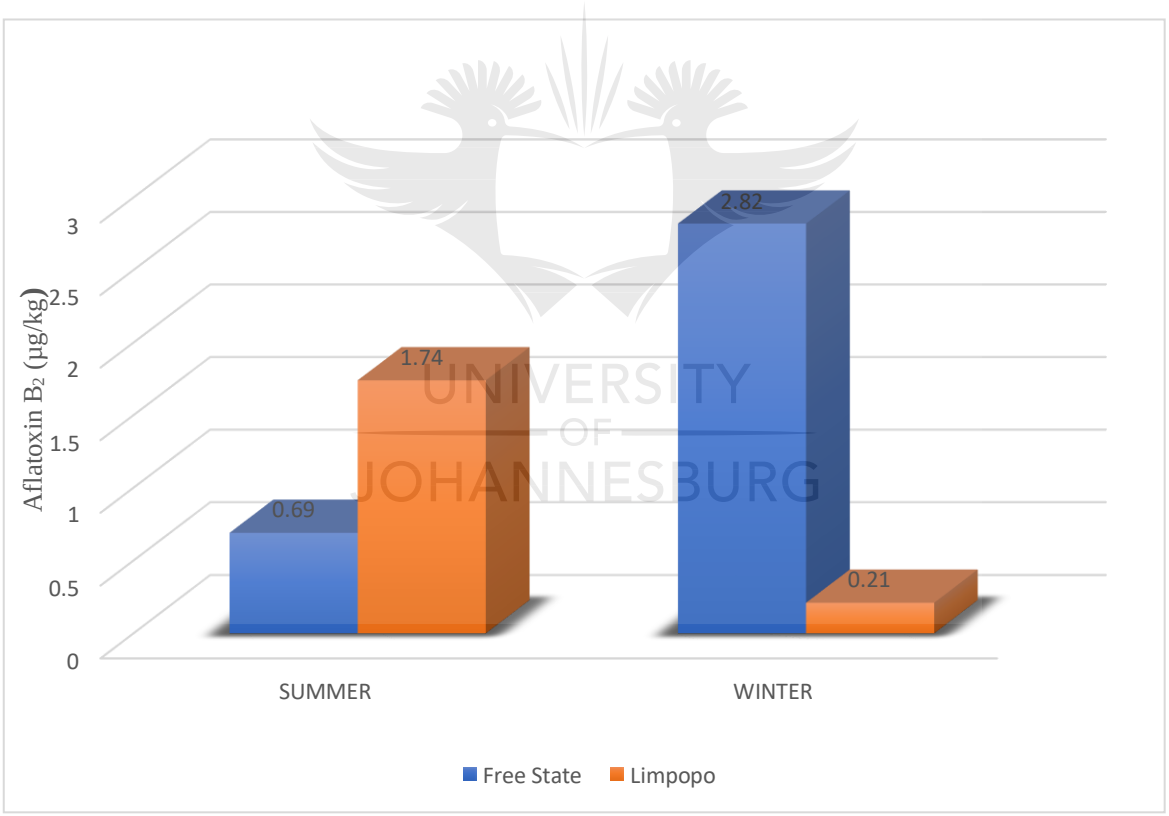


Figure 4.8: Mean concentrations of aflatoxin B_2 (AFB_2) produced by *Aspergillus flavus* isolated from dairy cattle feeds and feedstuffs from Free State and Limpopo provinces, South Africa.

It can be observed in Table 4.11 that all the single factors, including their Interaction have no significant effect (P > 0.05) on ZEN production by *Fusarium* isolates *(F. equiseti and F. oxysporum).* However, season had the highest effect (VR = 2.246) among the three tested factors. Figure 4.8 also revealed that the minimum mean concentration (9.34 µk/kg) of ZEN was produced by *Fusarium* isolates recovered from Free State winter samples, while the maximum concentration (35.08 µk/kg) was recorded in Limpopo summer samples.

Table 4.11: MANOVA for zearalenone (ZEN) production (µg/kg) by *Fusarium equiseti* and *Fusarium oxysporum* in dairy cattle feeds and feedstuffs in relation to season, province, and their interactions.

Effect	DF	MS	VR	P
Provinces	1	744.897	1.484	0.232
Season	1	1127.431	2.246	0.144
Province X season	1	957.504	1.907	0.177
Error	32	502.063		
Total	36			

DF = degree of freedom; MS = mean square; VR = variance ratio; P = probability at P ≤ 0.05.

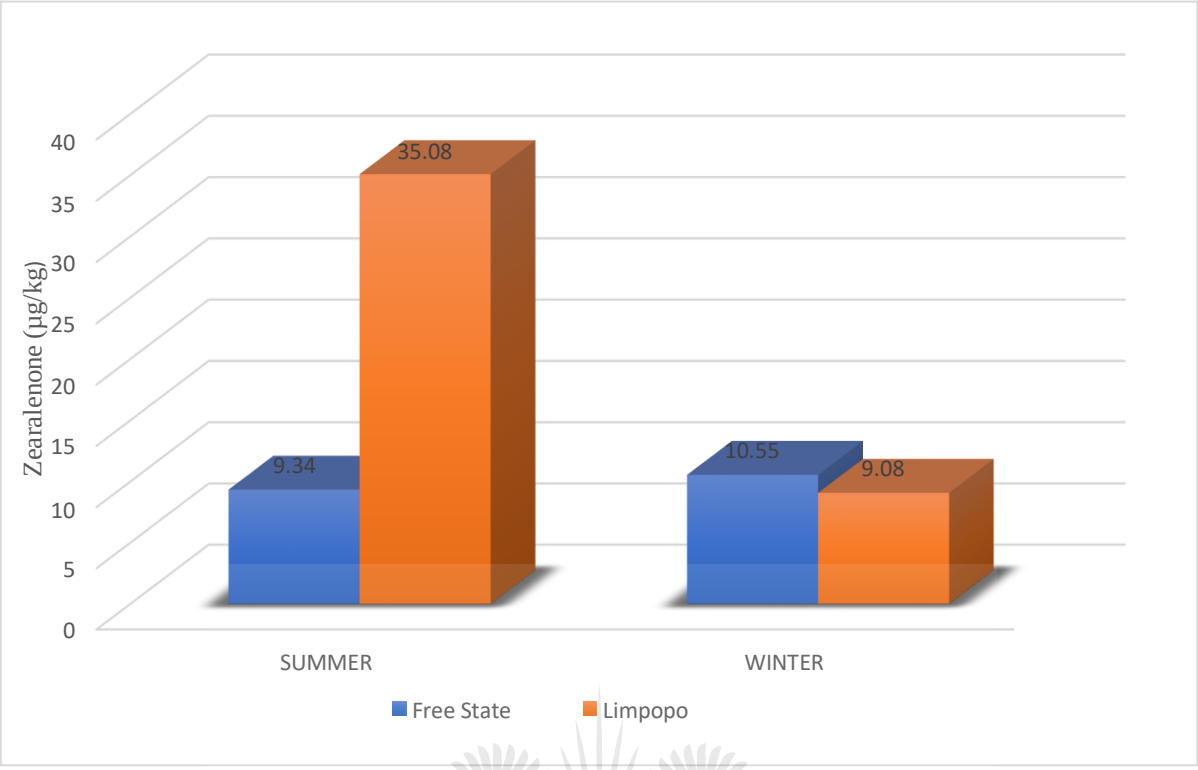


Figure 4.9: Mean concentrations of zearalenone (ZEN) produced by *Fusarium equiseti* and *Fusarium oxysporum* isolated from dairy cattle feeds and feedstuffs from Free State and Limpopo provinces, South Africa.

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

Contamination of animal feeds by fungi is a global problem because of the toxins they produce, which can be hazardous to animals and humans and have a severe influence on the economy of any country (Lacey *et al*., 2015). In sub-Saharan Africa (SSA), fungal contamination contributes massively to food and feed losses (Udomkun *et al*., 2017). As a result, there is a growing need in South Africa for better feed management to assist in monitoring these moulds in livestock feeds and feed ingredients. To achieve this, the toxins present in feeds and feed components must be tested regularly.

5.2 FUNGAL ISOLATION AND IDENTIFICATION

The primary goal of this research was to isolate and identify the fungi spp. contaminating dairy cattle feeds and feed ingredients in smallholder dairy farms in Free State and Limpopo provinces of South Africa, as well as their propensity to produce mycotoxins. This study revealed that 68/70 (97%) of the feeds were infected with one or more fungal species. A total of 237 fungal isolates belonging to 14 genera were recovered during the mycological screening of 70 feeds and feed ingredients. Overall, the culturable fungi population of the species ranged from 1.1 x 10^3 to 3.0 x 10^6 CFU/g throughout the two provinces and among the feed samples (Appendix A). In addition, the minimum and maximum mean fungal loads ranged from 9.3 x 10^3 to 3.0 x 10^5 CFU/g in Free State silages and Limpopo TMR, respectively (Table 4.1). The low level of fungal contamination in silages could be attributed to the fermentation process during silage making. According to Adebiyi *et al*. (2019), fermentation aids in inhibiting and suppressing the growth of pathogenic and spoilage microorganisms, hence improving food and feed quality. This was confirmed by Ndlovu and Dutton (2013), when they isolated 100 fungal species from 82 corn silage samples and 172 isolates from just 21 chopped maize samples. Njobeh (2009) also reported that fermented food products such as cassava flakes and flour were the least infected by fungi spp. among food products from Cameroon.

The data from this study indicate that various storage and field fungi are associated with South African dairy cattle feeds and feed ingredients. The presence of toxigenic fungal isolates has been documented in several agricultural commodities such as rice, wheat, flour, corn, and Bambara groundnut (Tournas and Niazi, 2017; Olagunju *et al.*, 2018), and these fungi have been recognised as causative agents responsible for mycotoxin contamination of South African dairy cattle feeds (Kemboi *et al.*, 2020; Changwa *et al.*, 2021). In general, the incidence of the various isolated fungal genera showed the prevalence of *Aspergillus*, followed by *Fusarium and Penicillium*. This work agrees with the study of Dutton and Westlake (1985), who isolated different fungal genera from 800 livestock feeds (including compound feeds, cereals, silage, and hay) from South Africa, *Aspergillus* was the most prevalent genera isolated from the feed samples, followed by *Fusarium*. A survey conducted in Brazil by Sima *et al*. (2007) showed a high prevalence of *Aspergillus* species (42.5%) in 80 samples of brewers' grain used in dairy cattle feeding. A similar study conducted in Argentina by Pereyra *et al*. (2008) revealed 78% of corn silage was contaminated with *Aspergillus* spp. Among the *Aspergillus* spp. isolated in this present study, *A. fumigatus* and *A. flavus* have the highest incidence, and this was closely followed by *A. niger* (Tables 4.3 and 4.4). The findings of this present study are in line with Maenetje and Dutton (2007), who found *Aspergillus* spp. in barley, an important dairy feed in South Africa, with *A. flavus* (80%) the most prevalent fungal genera in the study. A Similar study conducted by Ndlovu and Dutton (2013) revealed 15 *Aspergillus* spp. in maize silage and chopped maize (common dairy cattle feed), with *A. fumigatus* and *A. flavus* as the most prevalent fungal species occurring at incidence rates of 32 and 21 %, respectively.

Contamination of dairy cattle feeds from both provinces with *A. fumigatus* and *A. flavus* as the most prevalent may be attributed to late harvesting employed by the farmers. Most of the dairy cattle farmers leave their feedstuffs, especially cereals, for long on the farm sites with the possibility of fungal attack. The presence of fungal species in the feeds could also be explained by post-harvest conditions, including poor feed handling, improper storage facilities and conditions, as well as means of transportation. Kamika *et al*. (2014) revealed that fungal and mycotoxin contamination of agricultural products could be promoted by poor and longer storage conditions that favour fungal growth. This was the case during sampling when some of the dairy farmers

stored the feeds and feedstuffs destined for their cattle consumption in unhygienic environments and under conditions conducive for the growth of fungi. Contamination of dairy cattle feeds by fungi reduces feed quality, market value, and animal productivity, while also posing a health risk if the fungi can produce toxins such as AFs, FBs, DON, OTA, and ZEN (Kemboi *et al*., 2020). The co-occurrence of toxigenic fungi, as presented in this report, indicates how dairy cattle are exposed to these toxins with subsequent transfer to humans through consumption of by-products from animals that fed on such contaminated feeds. It was revealed from the phylogenetic analysis (Figures 4.2 and 4.3) in this study that most fungal species from the dairy feeds and feedstuffs showed a strong relationship with their relative species from the Gen Bank.

5.3 TOXIGENICITY OF FUNGI SPECIES ISOLATED FROM DAIRY CATTLE FEEDS AND FEEDSTUFFS FROM FREE STATE AND LIMPOPO PROVINCES, SOUTH AFRICA.

In general, dairy cattle feeds and feedstuffs were contaminated with fungi capable of producing mycotoxins such as AFB_1, AFB_2, as well as ZEN. The occurrences of toxigenic fungi genera, including *Aspergillus, Penicillium* and *Fusarium*, have earlier been reported in these substrates (Richard *et al*., 2007; Ndlovu and Dutton, 2013; Tangni *et al*., 2017), implying the existence of the mycotoxins identified. The *A. flavus* strains recovered in this study produced B-type aflatoxins (AFB_1 and AFB_2) but not G-type aflatoxins (AFG_1 and AFG_2). This study is similar to the work of Njobeh *et al.* (2009), where the isolated *A. flavus* produced only the B-type and not the G-type aflatoxins.

Strains of *A. ochraceus* recovered in this study did not produce OTA. It is possible that the synthetic medium (CYA) used in this current study is not suitable to produce OTA by *A. niger*. An earlier report indicated that the metabolic profile of fungal species depends on the growth medium, as well as the laboratory conditions (Chilaka *et al.*, 2012). Although 25 strains of *A. niger* were recovered in this study, none of them could produce OTA (Table 4.6). Munitz *et al.* (2014) similarly did not find OTA from 19 strains of *A. niger* isolated from Argentinian blueberry. In Africa, none of the *A. niger* strains isolated from Egyptian peanut tested positive for OTA (Sultan and Magan, 2010). This was similar to the work of Njobeh *et al*. (2009), where all

strains of *A. niger* recovered from 95 Cameroonian foods tested negative for OTA. This could be because the laboratory conditions are not suitable for OTA production by the fungus, or the isolates are not mycotoxigenic. However, our results for OTA production by *A. niger* differ from those obtained by Adekoya *et al.* (2018), who detected OTA in some South African fermented food products but at very low concentrations. Among the 6 *Fusarium* species recovered in this study, only two, *F. equiseti* and *F. oxysporum* were positive for ZEN. This could be because some of the *Fusarium* isolates are not mycotoxigenic.

It was noted in our study that none of the single factors tested (season or province) has a significant effect on AFB_1 production by *A. flavus*, and ZEN production by *F. equseti* and *F. oxysporum*, respectively. However, the season had the most effect among all the tested factors. Generally, the concentrations of AFB_1 (0.43 to 1045.4 µg/kg) and AFB_2 (0.13 to 3.44 µg/kg) detected in Limpopo were higher than the concentrations of AFB_1 (0.22 to 576.14 µg/kg) and AFB_2 (0.11 to 2.42 µg/kg) found in Free State, respectively. Moreso, the levels of AFB_1 (0.22 to 10445.8 µg/kg) produced during summer were higher than in winter (0.69 to 190.22 µg/kg). The same trend was observed for AFB_2 in the summer (0.11 to 3.44 µg/kg) and winter (0.21 to 2.82 µg/kg) (Appendix A). Also, 38% of the total AFs produced by the strains of *A. flavus* in this study exceeded the regulatory limits (10 µg/kg) set by the South African government for dairy cattle feeds and feedstuffs (Appendix B, Table 1). This study agrees with Alam *et al.* (2012), in which maximum concentrations of AFB_1 (191.65 µg/kg) in feeds were found during the summer season. Omotayo *et al*. (2019) also reported 98 and 96% of AFB_1 and AFB_2 in summer ginger compared to 86 and 56% recorded in winter ginger. In the same study, the concentrations range of AFB_1 (0.02 to 0.74 µg/kg) and AFB_2 (0.04 to 3.44 µg/kg) in summer ginger were lower than the concentrations range of AFB_1 (0.01 to 6.04 µg/kg) and AFB_2 (0.14 to 9.95 µg/kg) in winter ginger, respectively. *F. equiseti* and *F. oxysporum* recovered in this present work had previously been documented to produce ZEN elsewhere (Barros *et al*., 2012; Beev *et al*., 2013). This current report agrees with the study of Phoku (2014), however, the level of ZEN produced in this study was low, ranging from 5.20 to 97.18 µg/kg (Appendix B, Table 2) and below the South Africa acceptable level (500 µg/kg) in dairy feed (Kemboi *et al*., 2020). Moreover, ZEN has been reported to be more abundant in crops from North America, Western Europe, and Eastern Europe rather than Africa (Devegowda

et al. 1998).

The high level of mycotoxins, especially AFB_1, produced by the mycotoxigenic fungal isolates during summer could be due to poor agricultural practices, such as feed mishandling during harvesting, poor storage facilities and conditions where the moisture contents of the feed ingredients are not adequately regulated before storage and might promote fungal proliferation and mycotoxin production (Atanda, 2013). Moreso, climatic conditions in Limpopo, especially during summer, may be responsible for the high mycotoxin levels recorded in the region. Lastly, contamination of the feeds could have happened on the field when some of the feed ingredients used in the feed formulation were kept too long on the farm sites, giving a chance to invasion by fungi and subsequent mycotoxin production (Kaaya *et al*., 2006) or fungi vectors such as pests and insects (Avantaggio *et al*., 2002; Jeyaramraja *et al*. 2018).

5.4 CONCLUSION

This study evaluates the incidence of fungi in dairy cattle feeds and feedstuffs, their toxigenic potentials, as well as the effects of seasonal and geographical variations on the mycotoxigenicity of the fungal species. The presence of *Aspergillus*, *Fusarium*, and *Penicillium* isolates in the feeds coupled with their ability to produce certain mycotoxins, including aflatoxins (AFB_1 and AFB_2) and zearalenone (ZEN), necessitate the need for regular assessment of the mycological and mycotoxin profiling of South African dairy cattle feeds and feed ingredients. It is important to mention that certain conditions such as improper feed storage, infestation by pests and insects, poor agricultural practices, climatic conditions, and lack of awareness among dairy cattle farmers about fungi and mycotoxins may be responsible for the high contamination levels recorded in the two provinces particularly, during summer. As such, possible measures such as proper storage facilities and good storage conditions, and good agricultural practices need to be adopted to tackle health-related problems as well as establishing surveillance programmes to limit health effects on dairy cattle and improve animal by-product quality.

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APPENDICES

Appendix A

Table 1: Diary farms visited, province collected from, number of feeds collected in each season, storage method employed by the farmers and the duration of storage.

Farms	Province	No of feeds collected in summer	No of feeds collected in winter	Storage method	Storage duration
Farm 1	Free State	1	1	Bags	< 1 month
Farm 2	Free State	1	3	Field, Storeroom	3 – 6 months
Farm 3	Free State	-	1	Field	< 1 month
Farm 4	Free State	3	3	Storeroom	< 1 month
Farm 5	Free State	-	1	Storeroom	< 1 month
Farm 6	Free State	2	3	Bags	3 – 6 months
Farm 7	Free State	1	3	Storeroom	< 1 month
Farm 8	Free State	1	1	Storeroom	< 1 month
Farm 9	Free State	2	4	Storeroom	< 1 month
Farm 10	Free State	2	4	Storeroom	< 1 month
Farm 11	Free State	1	2	Storeroom	> 6 months
Farm 12	Limpopo	1	1	Bags	< 1 month
Farm 13	Limpopo	1	3	Storeroom	< 1 month
Farm 14	Limpopo	1	2	Bags, Storeroom	< 1 month
Farm 15	Limpopo	1	2	Container	< 1 month
Farm 16	Limpopo	1	2	Bags, Storeroom	3-6 months

Farms	Province	Season	No of feed collected	Feed storage method	
Farm 17	Limpopo	-	1	Storeroom	< 1 month
Farm 18	Limpopo	2	3	Storeroom	< 1 month
Farm 19	Limpopo	1	1	Bags	3-6 months
Farm 20	Limpopo	1	2	Storeroom	< 1 month
Farm 21	Limpopo	1	3	Storeroom	< 1 month

< = less than; > = greater than

Table 2: Fungal contamination of dairy cattle feed and feedstuffs from Free State and Limpopo and Limpopo Provinces, South Africa.

SN	Sample I. D	Fungal source	Fungal name	Fungal load (CFU/g)
1	GF01	Soybean	*A. niger* *A. fumigatus* *A. terreus* *P. crustosum* *F. equiseti*	7×10^4
2	GF02	Soybean	*A. flavus* *A. niger* *F. chlamydosporum* *F. equiseti*	9×10^4
3	GF03	Grasses	*A. flavus* *A. niger* *P. crustosum* *Trichoderma atroviride*	1.7×10^4
4	GF04	Grasses	*A. flavus* *A. niger* *P. crustosum* *Epicoccum sorghinum* *Rhizopus solonifer*	4×10^4
5	GF05	Lucerne	*A. flavus* *A. niger* *A. fumigatus* *P. crustosum* *F. equiseti*	1×10^4
6	GF06	Lucerne	*A. flavus* *A. fumigatus* *A. terrues* *F. equiseti*	1×10^5
7	GF07	Soybean	*A. terreus* *Epicoccum sorghinum* *F. equiseti*	1.1×10^6
8	GF08	Lucerne	*A. flavus* *A. terrues* *Epicoccum sorghinum*	1.2×10^5

SN	Sample I. D	Fungal source	Fungal name	Fungal load (CFU/g)
			F. equiseti	
9	GF09	Grasses	*A. fumigatus* *Epicoccum sorghinum*	5.2×10^5
10	GF10	Lucerne	*A. niger* *Trichoderma atroviride*	8×10^4
11	HS01	TMR	*A. flavus* *A. niger* *A. terreus* *A. candidus* *Paecilomyces formosus* *Trichoderma atroviride*	1.2×10^4
12	HS02	TMR	*A. flavus* *A. niger* *A. fumigatus* *F. oxysporum* *F. verticillioides*	5×10^4
13	HS03	TMR	*A. flavus* *A. fumigatus* *F. oxysporum* *F. chlamydosporum* *Trichoderma atroviride*	1.7×10^5
14	HS04	TMR	*A. candidus* *P. crustosum* *Epicoccum sorghinum* *F. chlamydosporum*	2.3×10^5
15	HS05	Dairy concentrate	*A. flavus* *A. ochraceus* *A. terreus* *F. verticillioides*	1×10^4
16	HS06	Grasses	*F. oxysporum* *F. verticillioides*	1.6×10^5
17	HS07	TMR	*A. niger* *A. fumigatus*	2.8×10^6

SN	Sample I. D	Fungal source	Fungal name	Fungal load (CFU/g)
			P. crustosum	
18	HS08	TMR	*A. flavus* *P. crustosum* *F. verticillioides*	2×10^4
19	HS09	TMR	*A. terreus* *F. oxysporum* *P. crustosum* *F. chlamydosporum*	3×10^6
20	HS10	TMR	*A. fumigatus* *Cladosporium cladosporioides* *F. incarnatum*	3.7×10^5
21	HS11	TMR	*A. flavus* *P. crustosum*	8×10^4
22	HS12	Pellet	*A. flavus* *Rhizopus solonifer*	6×10^4
23	HS13	TMR	*A. fumigatus* *F. oxysporum* *Cladosporium cladosporioides* *F. chlamydosporum*	4×10^4
24	HS14	TMR	*A. niger* *A. fumigatus* *F. oxysporum* *P. crustosum*	1.9×10^4
25	HS15	TMR	*A. niger* *A. ochraceus* *A. terreus* *Mucor plumbeus*	2×10^4
26	HS16	Lucerne	*F. oxysporum* *F. verticillioides*	4.1×10^5
27	HS17	TMR	*A. fumigatus* *Rhizopus solonifer* *F. chlamydospoum*	1.1×10^5

SN	Sample I. D	Fungal source	Fungal name	Fungal load (CFU/g)
28	HS18	TMR	*A. niger* *P. crustosum* *Cladosporium cladosporioides*	1.4 X 10^5
29	HS19	Dairy concentrate	*A. niger* *A. fumigatus* *Rhizopus solonifer*	1.1 x 10^4
30	HS20	Lucerne	*A. fumigatus*	5 x 10^4
31	HS21	TMR	*P. crustosum*	5.2 X 10^4
32	HS22	TMR	*A. flavus* *A. fumigatus* *A. candidus* *Alternaria alternata* *Rhizopus solonifer*	1 x 10^4
33	HS23	Silage	*A. flavus* *A. fumigatus* *Paecilomyces formosus* *Candida albican*	7 x 10^3
34	HS24	Silage	ND	ND
35	HS25	Silage	*A. flavus* *P. crustosum*	3 x 10^4
36	HS26	TMR	*A. fumigatus* *F. verticillioides*	2.4 x 10^5
37	JF01	Pellet	*A. flavus* *A. niger* *P. crustosum* *Rhizoctonia solani*	1.9 x 10^5
38	JF02	Grasses	*A. flavus* *Epicoccum sorghinum*	2.3 x 10^4
39	JF03	Soybean	*A. fumigatus*	1.6 x 10^5

SN	Sample I. D	Fungal source	Fungal name	Fungal load (CFU/g)
			Alternaria infectoria *Rhizoctonia solani* *F. equiseti*	
40	JF04	Lucerne	*A. niger* *Rhizopus solonifer*	2×10^4
41	JF05	Pellet	*F. oxysporum* *P. crustosum* *Rhizopus solonifer* *Talaromyces pinophilus*	6×10^5
42	JF06	Pellet	*A. flavus* *A. terreus* *A. candidus* *P. crustosum*	9×10^3
43	JF07	Pellet	*A. fumigatus* *A. terreus* *P. crustosum* *Alternaria alternata*	1.7×10^3
44	JF08	Pellet	*A. fumigatus* *A. terreus* *A. flavus* *Mucor plumbeus*	1.1×10^3
45	JF09	Pellet	*Alternaria alternata* *Rhizopus solonifer* *P. crustosum* *Talaromyces pinophilus*	3.4×10^4
46	NJ01	Grasses	*P. crustosum* *Mucor plumbeus*	5×10^4
47	NJ02	Pellet	*A. flavus* *A. niger* *A. terreus* *F. incarnatum* *Rhizopus solonifer*	3×10^4
48	NJ03	Lucerne	*A. fumigatus*	3×10^4

SN	Sample I. D	Fungal source	Fungal name	Fungal load (CFU/g)
			P. crustosum *Talaromyces pinophilus*	
49	NJ04	Ramilick	*A. flavus* *A. niger* *A. fumigatus* *Trichoderma atroviride* *F. brachygibossum*	1.7×10^5
50	NJ05	Silage	*Meyerozyma carribica* *Candida albican* *F. brachygibossum*	2.1×10^4
51	NJ06	Grasses	*A. flavus* *A. fumigatus*	4×10^4
52	NJ07	Lucerne	*A. fumigatus* *F. chlamydosporum*	1.3×10^5
53	NJ08	Pellet	*A. flavus* *A. niger* *F. equiseti* *F. oxysporum* *P. crustosum*	1.8×10^5
54	NJ09	Pellet	*P. crustosum* *F. chlamydosporum*	1×10^4
55	NJ10	Pellet	ND	ND
56	NJ11	Pellet	*A. niger* *A. fumigatus* *P. crustosum* *F. equiseti* *Trichoderma atroviride*	9×10^4
57	PD01	Soybean	*A. flavus* *A. niger* *F. incarnatum* *F. oxysporum*	1.4×10^5
58	PD02	Grasses	*A. fumigatus* *F. verticillioides*	1.2×10^5

SN	Sample I. D	Fungal source	Fungal name	Fungal load (CFU/g)
			Epicoccum sorghinum *F. chlamydosporum*	
59	PD03	Molasses	*A. flavus* *A. niger* *A. fumigatus* *P. crustosum* *F. brachygibossum*	4×10^4
60	PD04	Lucerne	*A. terreus* *F. oxysporum* *Alternaria infectonia* *Trichoderma atroviride*	1.2×10^5
61	PD05	Maize stove	*A. fumigatus* *F. chlamydosporum*	6.1×10^3
62	PD06	Dairy concentrate	*F. oxysporum* *Cladosporium cladosporioides* *P. crustosum* *F. chlamydosporum*	4×10^4
63	PD07	Dairy cocnentrates	*P. crustosum* *Meyerozyma carribica*	5×10^5
64	PD08	TMR	*A. niger* *A. fumigatus* *F. verticillioides* *Alternaria alternata* *Rhizopus solonifer*	5×10^4
65	PD09	TMR	*A. flavus* *Alternaria infectonia* *Epicoccum sorghinum* *Paecilomyces formosus*	8×10^5
66	PD10	TMR	*A. flavus* *A. fumigatus* *candida albican*	3×10^6
67	PD11	Lucerne	*A. fumigatus* *F. equiseti*	2×10^4
68	PD12	TMR	*A. fumigatus*	9×10^4

SN	Sample I. D	Fungal source	Fungal name	Fungal load (CFU/g)
			A. niger *F. equiseti*	
69	PD13	Molasses	*A. flavus* *A. niger* *A. fumigatus* *P. crustosum* *Alternaria alternata* *Paecilomyces formosus* *F. chlamydosporum*	1.3×10^4
70	PD14	TMR	*P. crustosum* *Rhizopus solonifer* *Trichoderma atroviride*	2.1×10^4

CFU/g = Colony forming unit per gram; TMR = Total Mixed Ration.

Appendix B

Table 1: Production of Aflatoxins (AFB1 and AFB2) by Aspergillus flavus extracts in dairy cattle feeds and feedstuffs from Free State and Limpopo Provinces, South Africa.

SN	Sample code	Fungal source	Season	Province	AFB_1	AFB_2	Total AFs
1	JF01	Pellet	Summer	Limpopo	1045.8	1.91	1047.71
2	GF05	Lucerne	Summer	Limpopo	2.16	ND	2.16
3	NJ08	Pellet	Summer	Limpopo	84.59	3.44	88.03
4	HS08	TMR	Summer	Free State	576.14	2.42	578.56
5	HS05	Others	Summer	Free State	0.38	ND	0.38
6	JF06	Pellet	Summer	Limpopo	1.15	0.13	1.28
7	GF08	Lucerne	Winter	Limpopo	2.95	0.21	3.16
8	JF08	Pellet	Winter	Limpopo	0.84	ND	0.84
9	GF03	Grasses	Summer	Limpopo	298.92	0.89	299.81
10	GF06	Lucerne	Summer	Limpopo	0.93	ND	0.93
11	HS23	Silage	Winter	Free State	0.69	ND	0.69
12	PD01	Soybean	Summer	Free State	0.80	0.11	O.91
13	GF04	Grasses	Summer	Limpopo	4.64	ND	4.64
14	NJ02	Pellet	Summer	Limpopo	0.43	ND	0.43
15	HS22	TMR	Winter	Free State	1.04	ND	1.04
16	NJ04	Others	Summer	Limpopo	18.85	0.75	19.6
17	HS12	Pellet	Summer	Free State	190.22	2.82	193.04

SN	Sample code	Fungal source	Season	Province	AFB_1	AFB_2	Total AFs
18	PD09	TMR	Summer	Free State	0.22	ND	0.22
19	HS01	TMR	Summer	Free State	47.34	0.11	47.45
20	NJ06	Grasses	Summer	Limpopo	2.36	ND	2.36
21	HS03	TMR	Summer	Free State	0.38	ND	0.38
22	PD13	Others	Winter	Free State	3.13	ND	3.13
23	PD03	Others	Summer	Free State	10.88	ND	10.38
24	HS11	TMR	Winter	Free State	14.44	ND	14.44

Others = ramilick and molasses; AFB_1 = aflatoxin B_1; AFB_2 = aflatoxin B_2; Afs = aflatoxins; ND = not detected. Concentrations of mycotoxins produced are recorded in µg/kg.

Table 2: Production of Zearalenone by *F. equiseti* and *F. oxysporum* extracts in dairy cattle feeds and feedstuffs from Free State and Limpopo Provinces, South Africa.

SN	Sample code	Fungal Source	Fungi Isolate	Season	Province	ZEN
1	PD06	Others	*F. Oxysporum*	Summer	Free State	12.52
2	JF05	Pellet	*F. Oxysporum*	Summer	Limpopo	7.80
3	HS02	TMR	*F. Oxysporum*	Summer	Free State	11.09
4	HS16	Lucerne	*F. Oxysporum*	Winter	Free State	15.90
5	HS03	TMR	*F. Oxysporum*	Summer	Free State	7.75
6	HS15	TMR	*F. Oxysporum*	Winter	Free State	5.20
7	GF06	Pellet	*F. equiseti*	Summer	Limpopo	97.18
8	GF08	Lucerne	*F. equiseti*	Winter	Limpopo	9.08
9	HS02	TMR	*F. equiseti*	Summer	Free State	8.69
10	JF05	Pellet	*F. equiset*	Summer	Limpopo	19.06
11	GF03	Grasses	*F. Oxysporum*	Summer	Limpopo	16.29
12	PD04	Lucerne	*F. equiseti*	Summer	Free State	7.64

Others = dairy concentrates; ZEN = zearalenone. Concentrations of mycotoxins produced are recorded in µg/kg.