AN INVESTIGATION INTO THE ANALYTICAL, CYTOTOXICITY AND IMMUNOTOXICITY OF MYCOTOXINS FOUND IN COMMERCIALLY AVAILABLE PELLETED PET FOODS IN DURBAN, SOUTH AFRICA

2018

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PREFACE

AN INVESTIGATION INTO THE ANALYTICAL, CYTOTOXICITY AND IMMUNOTOXICITY OF MYCOTOXINS FOUND IN COMMERCIALLY AVAILABLE PELLETED PET FOODS IN DURBAN, SOUTH AFRICA

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This thesis is submitted to the School of LMMS, College of Health Science, University of KwaZulu-Natal, Westville, for the degree of Doctor of Philosophy in Medical Biochemistry.

This is the thesis in which the chapters are written as a set of discrete research publications that have followed the Journal of the South African Veterinary Association format with an overall introduction, literature review and final summary. Typically, these chapters will have been published in internationally recognized, peer-reviewed journals.

This is to certify that the contents of this thesis is the original research work of Sanil Duleep Singh carried out under our supervision at the Mycotoxin Research Unit, Discipline of Medical Biochemistry, Howard College Campus, University of KwaZulu-Natal, Durban, South Africa.

Supervisor: Prof. Anil A Chuturgoon PhD.

Signed: ---------------------- Date: 18/03/2018

ABSTRACT

Introduction:

Dry pelleted dog food in the South African market is available via supermarket, pet stores (standard brands - SB) and veterinary channels (premium brands-PB). Similarly, cat food were viewed in two market segments.

Methodology:

Representative feeds from both categories were analysed for four main mycotoxins viz. aflatoxins (AF), fumonisin (FB), ochratoxin A (OTA), and zearalenone (ZEA) using standard well-described extraction, characterisation and quantitation processes.

Results:

All foods showed contamination with fungi (mainly *Aspergillus flavus, Aspergillus fumigatus* and *Aspergillus parasiticus)* and mycotoxins (the most prevalent being aflatoxins and fumonisins), irrespective of the brand. This study determined the immunotoxicity of extracts from pelleted dog and cat feed for mycotoxins. Isolated dog peripheral blood mononuclear cells (PBMCs) were treated with feed extracts to determine mitochondrial function, oxidative stress, and markers of cell death using luminometry and flow cytometry. Glutathione was significantly depleted by SB extracts. Markers of apoptosis and necrosis were elevated by both SB and PB feeds when compared to controls, with SB extracts being significantly higher than PB. ATP levels decreased with increased mitochondrial depolarization in cells that were exposed to both feed extracts with SB showing the greatest differences when compared to the control. Cat peripheral blood mononuclear cells (PBMCs) were isolated and treated with various feed extracts to determine oxidative stress (TBARS and GSH assay), mitochondrial integrity and cell death (Luminometry and Flow cytometry). Both PB and SB extracts showed significantly decreased ATP levels and increased mitochondrial depolarization except for the PB acid fraction. Lipid peroxidation was significantly increased in both PB and SB extracts with a concomitant decrease in GSH levels. Phosphatidylserine externalization and necrosis levels were increased in both PB and SB extracts when compared to the control. Executioner caspases-3/7 was also elevated following extract exposure except for the PB acid fraction.

Conclusion:

There were high levels of fungal contamination and mycotoxins in both categories of feed, regardless of the notion that higher priced PB's were of a higher quality.

DECLARATION I - PLAGIARISM

I, **Sanil Duleep Singh** declare that

1. The body of work reported in this thesis is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other person's writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
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Sanil Duleep Singh

DECLARATION II- PUBLICATIONS

1. A comparative analysis of mycotoxin contamination of supermarket and premium brand pelleted dog food in Durban, South Africa - In Press

Sanil D Singh and Anil A Chuturgoon

Journal of the South African Veterinary Association: Accepted 18 Aug 2017

Article Citation: 88 (0)a1488.https://doi.org/0.4102/jsava.v88i0.1488

2. A comparison of mycotoxin contamination of premium and grocery brands of pelleted cat food in South Africa –In Press

Sanil D Singh, Sooraj Baijnath, Anil A Chuturgoon

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3. Assessment mycotoxins extracted from contaminated dog pelleted feed on canine blood mononuclear cells – In Press

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4. The immunotoxicity of commercial pelleted feline feed in peripheral blood mononuclear cells (To be submitted)

Sanil D Singh, Alisa Phulukdaree, Naeem Sheik Abdul, Charlette Tiloke, Sooraj Baijnath, Anil Amichund Chuturgoon

Journal of the South African Veterinary Association

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To my mother, Thara Singh, who was ever present and whose quiet support was unequivocal.

LIST OF ABBREVIATIONS

AAFCO	Association of American Feed Control Officials
AF	Aflatoxins
AFB_1	Aflatoxins B_1
AFB_2	Aflatoxins B_2
AFG_1	Aflatoxins G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFM_2	Aflatoxin M_2
BEN	Balkan endemic nephropathy
BW	Body Weight
Cfu	Colony forming units
CIN	Chronic Intestinal Nephritis
CPA	Cyclopiazonic acid
CYA	Czapek yeast agar
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DON	Deoxynivalenol
EDTA	Ethylene diamine tetra-acetic acid
EU	European Union
FAO	Food and Agriculture Organization
FDA	Federal Drug Administration
FB	Fumonisin
FB_1	$Fumonisim_1$
FB_2	$Fumonisim_2$
FB_3	$Fumonisim_3$
HACCP	Hazard Analysis and Critical Control Point
HPLC	High Performance liquid chromatography
Kg	Kilogram
LD_{50}	Lethal dose to 50% of population
MEA	Malt extract agar
MTT	Methyl thiazole tetrazolium
OTA	Ochratoxin A
OTB	Ochratoxin B
PAT	Patulin
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PPE (PPO)	Porcine Pulmonary Edema (Oedema)
ppb	Parts per billion
ppm	Parts per million
RNA	Ribonucleic Acid
SAX	Strong anion exchange
TH	Trichothecenes
TLC	Thin layer chromatography
USDA	United States Department of Agriculture
ug	micrograms
ZEA	Zearalenone

TABLE OF CONTENTS

PREFACE I

ABSTRACT II

DECLARATION I - PLAGIARISM III

DECLARATION II- PUBLICATIONS IV

ACKNOWLEDGEMENTS V

LIST OF ABBREVIATIONS VI

TABLE OF CONTENTS I

Chapter 1-Introduction 1

Chapter 2- Literature Review 4
- Background 4
- Etymology and Concepts 4
- Hypothesis: 7
- Aim 7
- Objectives 7
- Research Consideration 7
- Fungal Contamination 10
- Fungal Genera 11
  - Aspergillus 11
  - Fusarium 11
  - Penicilluim 11
  - Alternaria 12
- Pet Food Recalls and Mycotoxin Outbreaks 12
- Common Feed Mycotoxins 14
  - Aflatoxins 14
  - Fumonisin 17
  - Ochratoxin A 20
  - Zearalenone 23
  - Alternaria 25
- Mycotoxin extraction 27
- Dog and Cat Immune System 28
- Acute and Chronic Toxicity 29
- Immune Disruptors 29
- Challenges -Regulation and Quality control 31
- References: 32

Chapter 3 - Paper 1 39

A comparative analysis of mycotoxin contamination of supermarket and premium brand pelleted dog food in Durban, South Africa 39

Abstract 40
Introduction 41
Materials and Methods 43
Results 46
Discussion 48
Conclusion 53
Acknowledgements 54

Chapter 4 - Paper 2 57

A comparison of mycotoxin contamination of premium and grocery brands of pelleted cat food in South Africa 57

Abstract 58
Introduction 59
Materials and Methods 60
Methodology 60
Results 61
Discussion 65
Conclusion 67
References 68

Chapter 5-Paper 3 70

Toxicity Assessment of Mycotoxins Extracted from Contaminated Commercial Dog Pelleted Feed on Canine Blood Mononuclear Cells 70

Abstract 71
Introduction 72
Materials and Methods 74
Results 78
Discussion 82
Conclusion 87
References 88

Chapter 6 - Paper 4 91

The immunotoxicity of commercial pelleted feline feed in peripheral blood mononuclear cells 91

Abstract 92
Background 93
Methods 94
Results 98
Discussion 102
Conclusion 106
Acknowledgements 106
References 107

Chapter 7-Conclusion 110

Future recommendations 112
References 113

Appendix 1- Animal Ethics Approval 114

Appendix 2 – Fungal Contamination **115**
Appendix 3 – Mycotoxin Analysis of Cat and Dog Samples **119**
Appendix 4 – Dog Cytotoxic Assays **124**
Appendix 5 – Cat cytotoxic assays **128**

Chapter 1-Introduction

The 21st century has brought many changes in attitudes towards companion animals, especially dogs and cats. The dissolution of the joint family, an increase in single living and nuclear families, have resulted in the increase of pet ownership in South Africa, which mimics an international trend. The global pet food market was estimated to be US $58.6 billion in the year 2011and is expected to grow to the US $74.8 billion in 2017. An increased awareness of animal welfare amongst pet owners, has resulted in pet food companies placing more emphasis on nutrition for different life stages. This has been coupled with convenience to suit modern living in confined spaces of apartments and townhouses. Hereby, resulting in the increased popularity of dry pelleted and extruded pet foods. Dry pet food accounts for 39% of global use followed by treats (21%) and cans (7%). The growing awareness amongst the global population about pet health and resulting humanization, has changed the way pet food conglomerates look at this market (Pet Food Market, 2016). Svetlana Udusilvaia of the Euromonitor International Conference who focused on global pet food sales trends, noted that there was an annual average 3% growth in cat and dog food sales. She also noted that larger growth was expected in emerging markets like China, Brazil, India and Russia. (Pet Food Analysis, 2017). Hence, resulting in the usage of dry pelleted diets and the shift away from home cooked diets. Since 2013, supermarket distribution channels, account for 70% of the pet food sales in South Africa. This may be supported by the wide range of brands that are competitively priced .This evidence is well supported by involvement of major conglomerates like Proctor and Gamble, Colgate-Palmolive and Mars Foods that view this business as a growth area.

Companion animal ownership has evolved from being a peripheral addition to an integral part of modern human life. In the USA, the total cat and dog ownership is about 150 million and is worth $50 billion and this translates to about 65% of households owning at least one pet. This figure is expected to exceed $62.75 billion and grow by 4-5% per annum (Pet Care Analysis, 2017). This trend stands good for most of the globe with China and India been the mushrooming markets. This trend of humanization has driven the pet food market forward as humans seek higher quality foods and accessories for their companion animals. Companion animals have graduated from being “outside pets” to being members of the household, living amongst human amenities. (Pet Food Market, 2016)

The main constituent of commercially dry pelleted pet food ranges are: 10-30 percent vegetable protein source, from about 20-60 percent amylaceous ingredients, from about 5-15 percent fat, and from 5-25 percent animal protein source depending on the individual feed formulation (Klich and Pitt, 1988, Dzanis, 1994). Soybean oil meal, soybean flour, soy protein concentrate, soy protein isolates, cottonseed meal, cottonseed flour, cottonseed protein concentrates, cottonseed protein isolates, peanut meal, peanut flour, peanut protein concentrates, peanut protein isolates, corn germ, corn germ meal, wheat germ, wheat germ meal, wheat gluten, corn gluten meal, corn gluten feed, corn distiller's dried grains, dried corn distiller's solubles make up the vegetable protein source. In addition, "amylaceous ingredients" consist of a preponderance of starch and/or starch-like material which include cereal grains and meals or flours obtained upon milling cereal grains such as corn, oats, wheat, milo, barley, rice, and the various milling by-products of these cereal grains such as wheat feed flour, wheat middlings, mixed feed, wheat shorts, wheat red dog, oat groats, hominy feed, and other such material. Sometimes, additional inclusion maybe tuberous foodstuffs such as potatoes, tapioca, various edible grain or tuberous starches and modified starches. These formulations provide an ideal substrate for fungal opportunistic contamination and growth. Cheaper pet food formulations have a higher cereal or cereal by product content, therefore, making these products more price accessible to the consumer, but with high potential for fungal contamination . Premium pet food formulations tend to have less of a cereal content but have higher animal source protein. However, these products are not insular to opportunistic fungal infection. It may be theorised that opportunistic fungi may thrive in high nutrient environment. (Boermans and Leung, 2007)

Ergotism was known from middle ages as a condition that arises from the consumption of mouldy food. However, not all strains of mould are capable of causing illness. Certain occurrences in the farming and veterinary industry in the late 50's and early 60's brought impetus to mycotoxins research. The first being the discovery of mould called *Pithomyces chartarum* found commonly in the grazing of sheep in New Zealand that caused facial eczema in sheep. The second major advancement occurred in Great Britain with discovery of Turkey X disease (Razzazi et al., 2001). This created a major loss in the poultry industry with high morbidity and mortality. This was attributed to groundnut that was contaminated with *Aspergillus flavus* that produced a secondary metabolites called aflatoxin (Razzazi et al., 2001).

Subsequent to these discoveries many further instances of deaths in farm and other animals were attributed to mouldy feed and their secondary metabolites (Leung et al., 2006). The first instance of serious mortality amongst canines was recorded in the South Eastern United States in 1955. Contaminated corn was suspected as the likely aetiology of dog that died of Hepatitis X (Adams et al., 2004). In the last five decades there have been regular instances of commercial pet food (or contaminated feed) incriminated in mycotoxins outbreaks worldwide . The continued threat of mycotoxins over the last five decades marches on from the 1974 outbreak due to contaminated corn in Indian villages (Krishnamachari et al., 1975) to 2011 in Gauteng ,South Africa, wherein cheap commercial dry dog food was implicated in the killing of an estimated 220 dogs (Arnot et al., 2012b).

The world population continues to gallop at an alarming rate in the developing world, hence there is an increased demand for food. In the drive to feed the millions of hungry humans, there will be a parallel demand to farm feed animals, in their role of food production and food security. An increase in pet ownership will add further threat to food security. Consequently, an increased food demand will result in cost cutting exercises that will result in contaminated ingredients, entering the food chain for both man and animals. An increased food demand, with an inclusion of contaminated grain, results in emerging diseases which are a serious threat to man, animal and environment, hence, “One Health” (Dhama et al., 2013).

Chapter 2- Literature Review

Background

Fungi, major pathogens of plants and animals, produce secondary metabolites called mycotoxins. These mycotoxins, when ingested, produce a wide spectrum of toxic effects in both humans and animals that can result in high morbidity and or mortality (Gajęcka et al., 2004, Krishnamachari et al.,1975, Boysen et al., 2002). The range of fungi may be divided into primary pathogens or secondary (opportunistic) pathogens. Primary fungi will affect normal healthy animals and humans or secondary fungi infect immune compromised hosts. Our interest lies in the mycotoxin category that enters living systems via contaminated foods, availing opportunistic circumstances, to cause disease. For the purpose of this study, the focus will be on mycotoxins of veterinary importance particularly in companion pets (dogs and cats) which include aflatoxins, fumonisins, ochratoxin A and zearalenone (Leung et al., 2006, Boermans and Leung, 2007, Mwanza et al., 2013).

Etymology and Concepts

Mycotoxins came to the fore during the 1962 outbreak of Turkey X disease in Great Britain, with its origins from two Greek words "***mikes and toxicum*** "translating to fungus and toxin, respectively. These toxins are of low molecular weight that are produced as a secondary metabolites by fungi. Fungi are plant-like organisms that are ubiquitously found in the environment which include moulds, yeasts and mushrooms. Many of these fungi are known to produce mycotoxins under favourable conditions of temperature and humidity. They require an organic substrate to support their growth. Some of toxigenic fungi belong to the genera *Aspergillus sp., Penicillum sp. and Fusarium sp*. These genera are commonly implicated in most animal related mycotoxin outbreaks. These fungi produce toxins under conditions suitable to the substrate and environment that supports the growth of a toxigenic mould (Pitt, 2000). These mycotoxins have a wide range of effects ranging from disease to death in humans and animals, and do display overlapping toxicities amongst their heterogeneous groups.

Mycotoxins may be clinically classified as hepatoxins, neurotoxins, nephrotoxins and cytotoxins based on the tissue they attack, whilst biologists prefer to class them into generic groups such as mutagens, carcinogens or allergens, etc. The golden period of mycotoxicosis was between 1960-1975, where the majority of the mycotoxins (Pulina et al., 2014) were discovered. However, only a few mycotoxins may be relevant to the pet and veterinary industry. When scientists linked "Turkey X" disease to the death of turkey poults via contaminated groundnut meal, their suspicions were aroused by the secondary metabolites produced by fungi that may be incriminated (Blount, 1961). This study we will focus on aflatoxins, fumonisins, ochratoxin A and zearalenone, as the mycotoxins most frequently implicated in animal health, though trichothecenes are also important (Leung et al., 2006). A number of studies looked at nutritive composition of pet foods, digestibility and mycotoxin profiles but few have investigated the mycotoxin pathogenicity on animal tissue. The effects on mammals and mammalian tissue varies per mycotoxin notwithstanding that some species show high tolerance and resistance to some mycotoxins. The study spectrum of mycotoxins are so vast that scientific quality oscillates from superficial analysis to more intricate cellular and molecular studies (Bennett and Klich, 2003).

Companion animals in the context of this study will include dogs and cats. Cats are obligate carnivores (Zoran, 2002) and contrary to the assumption dogs are not. Dog meat protein requirements are less than those of cats and hence dog diets tend to be formulated with high levels of vegetable material and probably alludes to a reason amongst many for the frequency of aflatoxin outbreaks.(Mwanza et al., 2013) Wild cat's diet consist of fat and protein and minimal quantities of carbohydrate (Council, 2006). These general assumptions have formed the basis for companion animal feed formulation but economic factors have given rise to substituting more expensive meat proteins with vegetable base proteins and fillers to drive costs down.

The 21 st century human custodians go to extreme lengths to ensure that their pets have the best possible quality of life, which includes their diets. Feed formulation in the modern era becomes especially important considering the value of companion animals to humans and the importance of their well-being However, are veterinary brands of pelleted pet foods safer than those sold via grocery and supermarket retails segments? Most pet foods have a high

concentration of cereals, vegetables and their by-products. As a cost cutting exercise manufacturers often use ingredients unsuitable for human consumption in pet food production. These ingredients are often contaminated with fungal species that present a risk to animals that consume it. However, manufacturers of premium diets make the claim that superior quality ingredients are used. Premium diets are priced at a higher rate implying better quality and subsequently better animal welfare. There are several studies evaluating fungal contamination and mycotoxin content in pet foods (Scudamore et al., 1997a, Martins et al., 2003), but limited studies that compare premium diets with supermarket (grocery) diets. Furthermore, little work has been done, correlating fungal contamination, mycotoxins (acid, neutral and acid neutral extraction fractions) and their cytotoxicity on the immune systems of dogs and cats. Furthermore, there are no studies testing mycotoxin extracts from premium (PB) and supermarket brands (SB) foods of both cat and dog on isolated peripheral blood mononuclear cells (PMBC) from the respective species. Mycotoxins are known to exert adverse health effects in animals and humans. The toxic effects vary depending on the chemical structure of the mycotoxin, mycotoxin concentration present in foods and feeds, and length of exposure (acute or chronic) (Fink-Gremmels, 1999). The immunosuppression and resulting genotoxic and cytotoxitc effects have been well described in the aetiology of human cancers with certain mycotoxins such as aflatoxin, ochratoxins and fumonisins (Zain, 2011). This implies that the focus in mycotoxin studies should centre around cytotoxic and mechanistic investigation rather than only on an analytical focus.

Hypothesis:

Premium (veterinary brand -PB) pelleted pet foods are less contaminated with fungi and hence have a lower content of mycotoxins and lower level of toxicity than those sold via supermarket (SB) retail channels.

Null Hypothesis:

There is no significant difference in the mycotoxin content or toxicity of both premium and supermarket feed brands.

Aim

To determine both the fungal and mycotoxin content of commercial pelleted (extruded) pet food in the Durban, KwaZulu-Natal, South Africa retail market in two marketing channels viz. PB and SB and their immunotoxic effects on PBMC's from both canines (dogs) and feline (cats).

Objectives

1. To identify and quantify fungal contamination of PB and SB pelleted pet food for both dogs and cats.
2. To extract and quantify mycotoxins that are commonly implicated in animal toxicities, viz., aflatoxins (AFB_1 and AFB_2), Fumonisins (FB_1 and FB_2), Ochratoxin A (OTA) and Zearalenone (ZEA) for both dogs and cats.
3. To determine cytotoxicity/immunotoxicity of PB and SB extracts/fractions (acid (A), neutral (N) and acid-neutral (AN)) on healthy PBMCs isolated from both dogs and cats.

Research Consideration

Complete blinding in this study is not possible but efforts to ensure maximum possibility was implemented. The sampling and analysis was conducted by different laboratory personal. Samples were encoded to ensure that actual product or segment was unknown to analytic personal. However, final data was analysed by the author in order to answer the research question.

Global and Local Pet Food Industry

Companion pets in the 21st century may include dogs, cats, rodents, birds, snakes and fish amongst others. In order that these pets thrive, they need to be fed a balanced and nutritive diet. Pet nutrition either comprises ingredients of a plant or animal source and or combination of both. The choice of the diet is dependent upon the owner's lifestyle and budget, hence the diet may be a fresh food diet, cooked diet or commercially available diet (Pet Food Market, 2016). In the past five decades, commercial diets have become more popular due to convenience, budget and aggressive marketing. Lifestyle changes, greater disposable income and awareness of animal welfare have changed the way people consider their pets. They provide companionship for people living a single lifestyle, replacing children in the empty nest scenario particularly with geriatrics. Coupled with these changes, have come changes in formulation, packaging and marketing of pet food resulting in major conglomerates entering this sector, that has been identified as a lucrative market. Four of the major international pet food producers are: Procter & Gamble, Nestlé, Mars, and Colgate Palmolive, which are thought to control 80% of the world's pet-food market amounting to US $58.6 billion in 2011 and is expected to rise to US $74.8 billion by 2017 (Pet Food Market, 2016).

The growth of pet food industry parallels urbanization associated with changing lifestyles and increasing economic resources and disposable income. To this there is a growing awareness of pet welfare, coupled with pet humanization, resulting in booming pet trade globally. Debbie Phillips –Donaldson the Editor-in-Chief of Pet Food Industry describes the US pet food market as "a bright future" that is substantiated with an anticipated capitalization of US $100 billion by 2020 from US $78 billion in 2015. This is attributed to 62% of American household owning pets, more purchases online, actively seeking safer foods and using social media to share their pet experiences (Pet Food Industry, 2017). Similarly the South African market has shown significant growth in spite of rising commodity prices, high unemployment and the devaluing of the South African Rand (ZAR). This is attributed to increased humanization and the rise in middle class income of the Black population. Supermarkets in South Africa continue to be the major marketing and distribution channels for pet food and products (70%) while speciality stores and veterinary clinics account for most of the remaining market. The speciality/veterinary channel is seen as a high end quality product. The South African market was valued in 2013 at ZAR 5 billion (US 550.7 million). Specialized brands (PB) accounted

for ZAR 1 billion, grocery brands (SB) ZAR 3.5 billion whilst non-grocery brands was ZAR 0.5 billion (Hundley, 2013).

Supermarkets often display numerous varieties of pet food for all life stages of the pet: puppy, young adult, mature, senior and even lactating or pregnant. The veterinary clinic and veterinary retail outlets are equally aggressive in their marketing of numerous feed variants and formulations. The veterinary market segment is seen as premium that is of better quality at a higher price. The formulation is perceived to have less vegetable matter than the supermarket brands. Similarly it is perceived to have higher amounts of protein from meat sources. Each life stage has its' own formulation, while each brand has its' own individual recipe. There are minimum standards that commercial pet foods must all adhere to, standards that are regulated by the Association of American Feed Control Officials (AAFCO) in USA, Commission Regulation (EU) No 107/2013 for the European Union, or governmental organisations, as in South Africa, in terms of the Fertilizer, Farm Feeds, Agricultural Remedies and Stock Remedies Act No.36 of 1947 (South African Government, 2009). However, these regulations say very little about digestibility or fungal contamination levels. Formulations of commercial pet foods are generally based on previous nutritional research. The Mars conglomerate which is one of the big four in the global pet food arena, funds the Waltham Centre for Pet Nutrition, which conducts scientific research into cat and dog nutrition and wellbeing (Burger, 1993). These research findings are publicly available in peer-reviewed journals but feed contamination remains a largely neglected area of study.

Climate change has also been a great influencing factor in commodity prices. Consequently, concerns of food security due to effects of climate change and shortages have driven food prices upwardly. In addition, the increase in pet ownership and increased demand for pet food have forced pet food companies to re-visit their feed formulations in order to keep prices competitive and affordable. This has resulted in companies often substituting expensive animal protein, with plant based protein and animal by-products thereby increasing the potential fungal contamination risk. The high percentage of plant based nutrients, incorporated into the feed formulation will increase fungal risk of mycotoxins to the animals (Boermans and Leung, 2007). At the same time, the addition of animal by-products, vitamins and food substrates may play a role in supporting fungal and bacterial growth under opportunistic circumstances. This ideology may apply to both PB and SB feeds. Cats unlike dogs are obligate carnivores and require fresh protein to be incorporated into their diets to avoid numerous diseases. The amino

acid taurine which is found in meat, is a dietary must for cats to avoid long-term taurine deficiency that may result in retinal degeneration, loss of vision, and cardiac arrest (Knopf et al., 1978).

In order for the pet food industry to combat mycotoxin contamination, a few processes need implementation. Some companies have added chelating agents that stabilize the mycotoxins and help reduce their impact on toxicity. Implementation of quality control and the use of Hazard Analysis Critical Control Points (HACCP) principles are reducing the impact of mycotoxins (Kabak et al., 2006). The use of human grade feed ingredients, improved packaging and long shelf life may reduce the impact of ill health in pets. Recent developments in mycotoxin resistant genetically modified maize may present an new opportunity for the future pet food formulation and its safety (Thakare et al., 2017).

Fungal Contamination

Fungi are plant like organisms that are ubiquitous, free of chlorophyll that include moulds, yeast and mushrooms which are capable of populating a myriad of surfaces and environments. They are largely saprophytic in nature and have well adapted hyphae that are capable of penetrating most surfaces and extracting nutrients for growth. More than 70,000 species of fungi have been recorded and described but not all are of economic importance. The species recognised as plant pathogens are of a filamentous nature that include *Aspergillus, Alternaria, Fusarium* and *Penicillium;* these fungal species are known to produce secondary metabolites called mycotoxins.

Further, these fungi commonly contaminate feeds and fodder and can pose a potential danger under environmentally favourable conditions of temperature and humidity (30^0C and 80 % or greater). The presence of visible spores on feed is an indication of some level of mycotoxin contamination.

The gold standard methodology employed for fungal isolation will be done by serial dilution according to Kaufman *et al.* (1963) followed by sub-culturing of isolated colonies on PDA, malt extract agar (MEA) and Czapek yeast extract agar (CYA) with subsequent macro- and microscopic identification. Determination of each species of fungi was done using the keys of

Klich and Pitt (1988) and Klich (2002) for *Aspergillus* spp. and Pitt and Hocking (1997) for *Penicillium* and other genera will be used to determine of each species of fungi.

Fungal Genera

Aspergillus

There are more than 180 species of *Aspergillus* but less than 10% of them are regarded as pathogenic. The most important members of this group are *A.flavus* and *A.parasiticus* that produce aflatoxin and have been implicated in aflatoxicosis. The 1959 outbreak of Turkey X disease implicated ground nuts contaminated with *A.flavus* (Blount, 1961). Other important members of this genus include *A. fumigatus, A.niger, A.ochraceus* amongst other less common species. This genus may also produce OTA (*A.niger and A.ochraceus)* with PAT produced by *A.clavatus*.

Fusarium

First discovered in 1809, this filamentous species is widely distributed in plants and cultivated soils hence often considered a field fungi. They usually opportunistically invade fruits, vegetables and grain prior to harvest with the common species being *F.solani, F.graminearum, F.chidosporuim, F.oxysporum and F.verticilliodes* amongst many economically less important species. *Fusarium* mycotoxins such as trichothecenes are most frequently described, implicating *F.verticilliodes* that most commonly found in animal diseases (Voss et al., 2007, Antonissen et al., 2014) and human heath (Chuturgoon et al., 2014, Bryden, 2007). *F.verticilloides* is known to produce fumonisin B_1, B_2 and B_3 which when ingested may give rise to a number of adverse health issues including long term carcinogenic outcomes.

Penicilluim

The Penicilluim species is most common fungus known to man. They are filamentous, opportunistic and have wide a range of habitats with over 150 accepted and recognised species (Pitt, 1989). By nature they are mostly soil dwelling and opportunistically enter the food chain

with cereals appearing to be the food product most affected. They are also associated with specialised fruit destroying pathogens eg. *P.digitatum, P.expansum and P.italicum* amongst many less common species. They are able to produce a range of mycotoxins that include OTA, PAT, Citrinin and Penicillic acid (Pitt and Hocking, 1997).

Alternaria

This fungal genus has had limited data but is recognised as a plant pathogen of some economic importance (Dutton and Kinsey, 1995). They produce tenuazonic acid and alternariol (King et al., 1979) and may be produced on a wide range of foods. Alternaria has a characteristically large brown club shaped conidia with separations that maybe transverse and longitudinal (Pitt and Hocking, 1997). Compared to *Aspergillus, Penicilluim and Fusarium, Alternaria* are significantly less pathogenic hence limited research interest in the genus.

Pet Food Recalls and Mycotoxin Outbreaks

In 2007 there was a massive recall of many brands of cat and dog foods. This recall was as a result of many consumer complaints with regards to their pet's ill health (Brown et al., 2007). This became a global phenomenon with deaths of cats and dogs being reported from all over the North American continent and even South Africa. Animals revealed primarily kidney failure as a result of melamine contaminated wheat gluten sourced from China (Puschner and Reimschuessel, 2011). Within a month of the outbreak, there were many pet deaths amongst nearly a thousand reported cases of kidney pathology (Brown et al., 2007). Poor records, both globally and locally, as regards actual number of affected pets may never be known and experts are concerned that the actual death toll could potentially reach into the thousands. Aon, a global provider of risk management solutions, revealed that pet illness and death numbered 1950 cats and 2200 dogs. Furthermore, more than 60 million containers of pet food was recalled resulting in $42 million in direct losses not withstanding indirect losses due to sales (Maberry, 2016). This incident changed pet food company's 'attitude towards the consumers' and animal welfare with more stringent quality control measures implemented. However, they have still not placed enough attention to fungal contamination which has resulted in numerous mycotoxin outbreaks in the companion and animal feed industry.

Prior to discovery of Turkey X disease (Blount, 1961) little was known about fungal contamination and toxicities. Among the earliest cases of food contamination and poisoning in dog feed was recorded in 1951-1955 in South-eastern USA, wherein 71 cases were recorded with many dogs dying. A commercial brand of dog food was implicated (Bailey and Groth Jr, 1959). In 1974 innumerable stray dogs and about 97 people died in an outbreak in Gujarat and Rajasthan, India, due to contaminated corn. Analysis of the corn revealed between 6.25 -15.6 mg of aflatoxin (Krishnamachari et al., 1975). Typically doses of 0.5-1 mg/kg body weight (BW) are enough to elicit serious clinic symptoms of anorexia, vomiting, lethargy, jaundice and subsequent death (Böhm et al., 2010). Further cases of mycotoxicosis were recorded between 1975 -1990 in USA (Liggett et al., 1986), Germany (Leung et al., 2006), South Africa (Bastianello et al., 1987) and Australia (Hocking et al., 1988). The primary incriminate was AFB1 whilst cases in Germany implicated ochratoxins with concurrent herpes virus infection. Penitrem was implicated in Australian cases and mouldy hamburger buns, being the aetiological cause. The 1987 South Africa mycotoxin outbreak was the result of a brand of contaminated commercial food which led to the death of 10 dogs. The period of 1991-2006 recorded many further cases across the globe. Cases were recorded in United Kingdom (Little et al., 1991), USA (Boysen et al., 2002, Stenske et al., 2006, Garland et al., 2007) and Korea (Jeong et al., 2006) with aflatoxin being most commonly implicated mycotoxin and penitrem, OTA and deoxynivalenol to a lesser extent. In eastern USA (2005), the deaths of a large number dogs were attributed to 19 different commercial diets related to contaminated corn, however, the mycotoxin implicated was not determined (Stenske et al., 2006). These cases may be attributed to a mycotoxin mix resulting in synergism, which is described as being, that the combined effect of mycotoxins are much greater than the sum of the effects of each mycotoxin alone (Eaton and Klaassen, 1996). The outbreak of canine aflatoxicosis, in Gauteng South Africa in 2011, saw more than 100 dogs being presented at the Onderstepoort Veterinary Academic Hospital (Pretoria, South Africa), with clinical symptoms associated with feed related toxicity. These were associated with three low cost brands of pelleted dog foods sold in the region. Many owners indicated that other dogs in the household died after been enticed to eat the food with the addition of gravy or meat when they initially resisted its consumption (Arnot et al., 2012b).

The USA summer of 2015 saw a number pet food recalls. In total, at least 28 pet food recalls were issued last year (2016), according to *Food Safety Magazine*'s own tally. *Salmonella* contamination constituted 35% of recalls while *Listeria*, along with low levels of

thiamine (vitamin B1), high levels of vitamin D and the presence of mould or propylene glycol constituted the remaining 65%. A consumer funded study referred to as the Pet Food Test (the first consumer funded study), was in-depth screening of pet food and its contents. Twelve pet food products of 6 cat foods and 6 dog foods were screened comprehensively for bacterial content (9 contained one or more bacteria or "qualifying pathogens;" 10 contained one or more "pathogenic microorganisms;" 9 contained one or more bacteria linked to spoilage of meat; 9 contained one or more potential pathogenic bacteria), cyanuric acid melamine, euthanizing drugs, and nutrient and mineral content [(4 contained excessive nutrient levels compared to standards set by the National Research Council and the American Association of Feed Control Officials (AAFCO). Due to financial and budgetary limitations, only 8 of the 12 pet foods were tested for 37 different mycotoxins resulting in 2 high risk, 2 medium risk and 4 low risk samples being reported.

Though pet food recalls continue to pop up at regular intervals, great strides have been made by responsible producers in the industry. An improvement in pet food manufacture, better quality control and upgrading of their facilities, have ensured better finished products and forcing companies to maintain honesty . However, pet food recalls will continue to occur with the presence of predatory low cost manufactures, global feed ingredients shortages, coupled with competition for resources in ensuring food security for humans.

Common Feed Mycotoxins

Aflatoxins

Figure 1. Chemical structure of Aflatoxin B_1 and B_2 ((http://www.chemspider.com/Chemical-Structure.2006507.html)

Aflatoxins are secondary metabolites which are difuranocoumarin derivatives of the polyketide pathway of mainly *A. flavus* and *A. parasiticus*. The outbreak of Turkey X disease due to the consumption of contaminated peanut meal resulted in the deaths of over 100,000 turkey poults (Blount, 1961). This diabolical incident provided the platform for investigative and diagnostic work in the area of mycology which resulted in the isolation and characterisation of the secondary metabolite aflatoxin (Allcroft et al., 1961). Additionally, Aflatoxins similarly to ochratoxins, often contaminate grain stored under poor conditions with inadequate drying or overheating. Subsequently, four aflatoxins were isolated named B_1 (figure 1), B_2 (Figure 2), G_1 and G_2 which was based on their fluorescent response to UV light.

Aflatoxins commonly affect groundnuts and less often other cereals. Aflatoxins are strongly associated with toxicity and death amongst animals, besides being an important carcinogen (Eaton and Groopman, 2013, Wogan, 1973) in both animals and humans. Aflatoxin B_1 is the most potent of all its varieties. The potent hepatotoxic mycotoxin, after been absorbed through the gastrointestinal tract is transported in the blood via hepatic portal system; thus, the liver becomes the primary organ affected (McLean and Dutton, 1995). Infected crops are often contaminated by *Aspergillus sp.* in the field and this condition is exacerbated by poor storage conditions, inadequate drying and overheating, especially in wet harvesting crops. Aflatoxin is probably the best known and most intensively researched mycotoxins in the world that have been associated with various diseases in livestock, companion animals and humans. As early as 1951, the deaths of dogs were associated with contaminated commercial food (Bailey and Groth Jr, 1959, Newberne and Butler, 1969) and periodically repeated itself during the last 70 years. The 2011 South African aflatoxicosis outbreak resulted in over 100 deaths in dogs (Arnot et al., 2012b). Dogs exposed to high levels of AF (> 1mg/kg BW) die within a few days with acute liver pathology (Lazicka and Orzechowski, 2010, Arnot et al., 2012b). Canines exposed to low levels 9<0.5-1 mg/kg BW) developed sub-acute symptoms characterised by anorexia, lethargy, jaundice and death within a few weeks of exposure (Newberne and Butler, 1969). Young animals and birds are more sensitive to aflatoxin toxicity. Among birds ducks and turkeys are particularly susceptible. Adult sheep and cattle are relatively resistant, while horses, pigs, rabbits and young calves are particularly sensitive to concentrations above 1mg/kg.bw. which are usually fatal for them. (Aquino and Corrêa, 2011, Naude et al., 2002). Bovines often consume aflatoxin contaminated feed with minimal toxic effects because they

metabolically bio-transform AFB_1 into a milder form called AFM_1. AFM_1 is often found in milk of lactating cows (Stoev, 2013).

The occurrence of aflatoxicosis is multi-aetiological and is influenced by location, environment, handling and storage of either grains, cereal or produced foods. Since a 100% safety of zero defect is a pipe dream, many government and non-government authorities have endeavoured to impose stricter regulations and limit exposure through quality control measures of input viz. Hazard Analysis Critical Control Points (HACCP) or ISO standards in manufacturing processes. Managing mycotoxin risk becomes a more realistic goal rather than mycotoxin free diets (Table 1).

Table 1. Present limits permissible of aflatoxin in animal feeds (FDA, 2001):

Crop type	**Animal feed**	**Permissible limit (ppb)**
Corn, peanuts and cottonseed meal	Cattle, pigs, poultry	200-300
Corn, peanuts and cottonseed meal	Breeding cattle, pigs, poultry	100
Corn, peanuts and cottonseed meal	Immature and other species	20

Fumonisin

FB2

Figure 3. Chemical structure of FB_1 (http://www.chemspider.com/Chemical-Structure.2015284.html).

Figure 4. Chemical structure of FB_2 (http://www.chemspider.com/Chemical-Structure.2015284.html).

The fumonisins are a group of mycotoxins that arise as secondary metabolites of *Fusarium sp.* species. They have strong structural similarity to sphingosines which is the backbone precursor of sphingolipids. Fumonisins can be further broken done into subtypes FB_1 (Figure 3), FB_2 (Figure 4) and FB_3 (of little relevance).

Fumonisin toxicity in mammals is associated with the feeding of mouldy maize, usually over a period of several weeks. Fumonisins are primarily produced by *Fusarium verticilloides* and *Fusarium proliferatum*. Conditions favouring fumonisin production appear to include a period of drought during the growing season with subsequent cool, moist conditions during pollination and kernel formation. Current evidence suggests that FB_1 and FB_2 are of similar toxicity, whereas FB_3 is relatively nontoxic (Stoev et al., 2009). Corn grain may commonly contain 1–3 ppm fumonisins, but occasionally as much as 20–100ppm are found. The toxins are concentrated primarily in mouldy or damaged corn.

Fumonisins are responsible for two well-described diseases of animals, equine leukoencephalomalacia (Marasas et al., 1988a, Thiel et al., 1991) and porcine pulmonary oedema (PPO) (Colvin and Harrison, 1992, Dilkin et al., 2003). Equine leukoencephalomalacia is a mycotoxic disease of the CNS that affects horses, mules, and donkeys which was first discovered in South Africa in 1970. It occurs sporadically in North and South America, South Africa, Europe, and China. Signs in equines include apathy, drowsiness, pharyngeal paralysis, blindness, circling, staggering, and recumbency. The clinical course is usually 1–2 days but may be as short as several hours or as long as several weeks. Icterus may be present when the liver is involved. The characteristic lesion is liquefactive necrosis of the white matter of the cerebrum; the necrosis is usually unilateral but may be asymmetrically bilateral. Some horses may have hepatic necrosis similar to that seen in aflatoxins. Horses may develop leukoencephalomalacia from prolonged exposure to <5mg FB_1/kg of feed, and the onset of neurologic signs almost invariably leads to death (Pienaar et al., 1981, Lioi et al., 2004). The liver and kidney remain the most susceptible organs, though prolonged chronic exposure has severe cytotoxic and immunosuppressive outcomes (Voss et al., 2007).

Fumonisins have also been reported to cause acute illness in weanling or adult pigs, characterized by pulmonary oedema and hydrothorax. Porcine pulmonary oedema is usually an acute, fatal disease and appears to be caused by pulmonary hypertension with transudation

of fluids in the thorax, resulting in interstitial pulmonary oedema and hydrothorax (Colvin and Harrison, 1992). This can be brought about by FB_1 concentrations as low as 20 ppm but not above 100 ppm as stated (Posa et al 2013, 2016; Kovacs et al 2016). Acute PPO results after consumption of fumonisins for 3–6 days at dietary concentrations >100 ppm. Morbidity within a herd may be >50%, and mortality among affected pigs is 50%–100%. Signs include acute onset of dyspnoea, cyanosis of mucous membranes, weakness, recumbency and death, often within 24 hrs after the first clinical signs. Affected sows in late gestation that survive acute PPO may abort within 2–3 days, presumably as a result of foetal anoxia. Prolonged exposure of pigs to sub-lethal concentrations of fumonisins results in hepatotoxicosis, characterized by reduced growth; icterus; and increased serum levels of cholesterol, bilirubin, AST, lactate dehydrogenase, and γ-glutamyltransferase (Colvin and Harrison, 1992). Pathogenesis for PPO or liver toxicities is believed to be due to fumonisin's ability, to interrupt sphingophospholipid synthesis and results in a series of disturbances in the dynamics of cardiopulmonary function leading to acute pulmonary oedema.

Cattle, sheep, and poultry are considerably less susceptible to fumonisins, than horses or swines. Cattle and sheep tolerate fumonisin concentrations of 100ppm with little effect. Dietary concentrations of 148 ppm in calves were symptomatically unaffected but did show changes in blood enzymes (Maenetje et al., 2008). Poultry are affected by concentrations of >200ppm and may develop poor appetite, weight loss, and skeletal abnormalities (Ledoux et al., 1992). Laboratory rodents' exposure to daily fumonisin dosing did develop renal and hepatic lesions; with rabbits being more susceptible then rats. However, there is limited and poor information on fumonisin toxicity in companion animals. The presence of a multi-mycotoxin mix in commercial dog food presents a high risk (Boermans and Leung, 2007).

No effective treatment is available for fumonisin toxicity which results in both high morbidity and mortality. Avoidance of mouldy corn through better quality control is the only prevention, although this is difficult because the corn may not be grossly mouldy or may be contained in a mixed feed. Limits allowed in milled dry corn and its various products is 2 - ppm. Permissible limits in animal feed is 5ppm for lab rodents, between 30-100ppm for livestock depending on species and their stage of production and 10ppm for companion animals (FDA, 2001).

Ochratoxin A

The genera *Aspergillus* and *Penicillum* which ubiquitously grows on a wide range of food commodities are known to produce a toxic metabolite under opportunistic circumstance called ochratoxin A (Figure 5).

Figure 5. Chemical structure of Ochratoxin (http://www.chemspider.com/Chemical-Structure.237890.html).

The ochratoxins are pentaketides made up of dihydro-isocoumarin linked to β-phenylalanine. OTA is more toxic than ochratoxin B, while ochratoxin C, α and β that are structurally related but are of little importance. This discussion will centre on OTA (Figure 5) since it is most relevant to animal and human health.

In tropical and sub-tropical regions, OTA is produced mainly by *Aspergillus* species, particularly the widespread *A. ochraceus*. But in temperate climates (Canada, Northern Europe and parts of South America), the main producer is *Penicillum verrucosum*. OTA production by *A. ochraceus* is favoured by relatively high temperatures (13°C to 37°C), but *P. verrucosum* grows and produces the toxin at temperatures as low as 0°C. *A. ochraceus* and *P. verrucosum* are opportunistic storage fungi, rather than field contaminants or plant pathogens. The production of the toxin occurs mainly when feeds are stored under inappropriate conditions, particularly with high moisture levels. OTA has been found in a wide range of raw and processed food commodities across the globe. It was first reported in cereals, but has since been found in other products, including coffee, dried fruits, wine, beer, cocoa, nuts, beans,

peas, bread and rice (Trucksess et al., 1998). It has also been detected in meat, especially pork and poultry, following transfer from contaminated feed (Speijers and Van Egmond, 1993).

OTA is a relatively heat-stable molecule and survives most cooking processes, although the reduction in concentration during heating depends on factors such as temperature, pH and other components of the product. For example, heating wet wheat at 100ºC for 2.3hours gave a 50% reduction in OTA concentration, but in dry wheat, the same reduction took 12 hours. OTA is destroyed by acid and alkaline hydrolysis and by the action of some oxidising agents (Omar, 2013). OTA levels in different food products vary, but are generally low in properly stored commodities (mean value less than 1µg/kg for cereals from temperate regions). However, much higher concentrations can develop under inadequate storage conditions. Levels of up to 6,000µg/kg and 5,000µg/kg have been reported in Canadian wheat and UK barley respectively, but the concentrations found are usually below 50µg/kg (Tolleson et al., 1996). The major contributors to OTA in the diet in Europe, are cereals and wine. Pork products have also been suggested as a significant dietary source (Speijers and Van Egmond, 1993).

OTA is a potent nephrotoxin and causes both acute and chronic effects in the kidneys of all mammalian species. The pathogenesis centres around the degradation of proximal tubules with subsequent nephritis (Maia and Pereira Bastos de Siqueira, 2002, Boysen et al., 2002). OTA is being described as a genotoxic, teratogenic and even carcinogenic in nature amongst many animal species. In acute toxicity trials, dogs and pigs appear most susceptible to LD_{50} of 0.2 - 1mg/kg BW while chickens (LD_{50} 3.3mg/kg BW) and rodents (LD_{50} 25-58mg/kg BW) appear more resistant (Naude et al., 2002). Studies have shown ability of OTA to induce oxidative stress and has a potential to modulate immune function resulting in cytotoxic outcomes (Bernardini et al., 2014, Hocking et al., 1988). An interesting clinical trial on beagle dogs recorded a wide range of symptoms for ochratoxicosis which included anorexia, vomiting, elevated temperature, purulent conjunctivitis, tonsillitis, polydipsia, polyuria blood stained rectal discharge, dehydration, prostration and death in the sequence listed (Szczech et al., 1973). This took about a fortnight to develop at a dosage rate of 0.2 -0.3mg/kg BW but dogs given 3-8.0mg/kg BW were dead within 48hours (Gourama and Bullerman, 1995). The LD_{50} for chicks was estimated to be 3.3-3.9mg/kg BW (Peckham et al., 1971) and for rats at 20mg/kg BW (Purchase and Theron, 1968).

OTA has been detected in human blood and breast milk, demonstrating dietary exposure through a wide range of contaminated food sources (Naude et al., 2002). The proliferation of the responsible fungus under unfavourable storage condition results in production of the secondary metabolite. The nephropathic diseases in human (Balkan Endemic Nephropathy (BEN)) that occurred in the Balkans in 1950, parallels the range of symptoms in pigs due to OTA toxicity (Antonissen et al., 2014). However, BEN is believed to be influenced by a multi-aetiology cause which is probably due to synergism amongst mycotoxins in the mix (Bennett and Klich, 2003).

The ability of OTA-producing fungi to grow on a wide range of food commodities its ubiquity presents a challenge in prevention control. This challenge is best achieved by measures designed to prevent the contamination of foods using HACCP-type tools. Detection and removal of OTA-contaminated material from the food supply chain is also important for imported products.

Few countries outside Europe have imposed limits for OTA, Uruguay sets a limit of 50µg/kg for rice, cereals and dried fruits and Canada sets a limit of 2,000µg/kg for OTA in pig and poultry feed (Lawley, 2013). No data is available on ochratoxin permissible limits in feed, in spite of research performed on companion animal with extracts (Szczech et al., 1973, Kitchen et al., 1977).

Zearalenone

Figure 6. Chemical structure of Zearalenone (http://www.chemspider.com/Chemical-Structure.129801.html).

Mycotoxins produced by *Fusarium* spp. may be the non-estrogenic trichothecenes, including deoxynivalenol, T-2 toxin, and diacetoxyscripenol, or the myco-oestrogens, including Zearalenone (ZEA) and zearalenol. ZEA and zearalenol are both oestrogenic resorcylic acid lactone compounds produced by the fungi *Fusarium* spp. as a secondary metabolite (Van Dongen and de Groot, 1995). ZEA is a macrocyclic lactone produced by *F. graminerium* and sometimes other Fusarium species and have a very high affinity for oestrogen receptors. They are capable of producing oestrogenic like symptoms with enlargement of teats and vulva, particularly in piglets (Van Dongen and de Groot, 1995). ZEA has been detected in a variety of cereals most commonly in maize and wheat, but can be redistributed during the milling process and hence ,may enter the food chain via other food products (Naude et al., 2002). It has been occasionally detected in samples from pastures in temperate climates at levels believed sufficient to cause reproductive failure of grazing herbivores. ZEA is of a low toxicity of about 1mg/kg feed that may cause many infertility symptoms particularly in young animals (Wannop, 1961, Minervini and Dell'Aquila, 2008).

ZEA and its metabolites, high affinity for oestrogen receptors increases the risk for reproductive abnormalities and disease in animals. The risk to humans has been demonstrated in women suffering from breast cancer and endometritis (Sharma, 1993).

Poultry appears to have some resistance to ZEA toxicity, but sheep and cattle may be periodically affected. Porcine and canines appeared to have the greatest susceptibility than other species (Richard, 2007).

Swine are affected with symptoms in pre-pubertal gilts including enlarged mammae, swelling of uterus and vulva, and atrophy of the ovaries. In severe cases, prolapse of the vulva and rectum may occur. Boars exhibit enlarge mammae and atrophied testes (D'mello et al., 1999) Clinical effects cannot be distinguished from excessive oestrogen administration. Physical and behavioural signs of oestrus are induced in young gilts, by as little as 1ppm dietary ZEA. There is hypertrophy of the mammary glands and uterus, and abdominal straining results in, prolapse of the uterus in severe cases. Removal of affected grain results in return to normal in 1 week (Minervini and Dell'Aquila, 2008). ZEA causes reproductive toxicities in sexually mature sows by inhibiting secretion and release of follicle-stimulating hormone (FSH), resulting in arrest of pre-ovulatory ovarian follicle maturation. Reproductive effects in sexually mature sows depend on time of consumption. ZEA fed at 3–10ppm on days 12–14 of the oestrus cycle in open gilts results in retention of corpora lutea and prolonged anoestrus (pseudo pregnancy) for up to 40–60 days. ZEA fed at ≥30 ppm in early gestation (7–10 days after breeding) may prevent implantation and cause early embryonic death. ZEA metabolites can be excreted in milk of exposed sows, resulting in hyper-oestrogenic effects in their nursing piglets (Long and Diekman, 1984, Chang et al., 1979).

Studies with dogs that were gavaged with 5mg/kg/BW of ZEA for 13 weeks revealed marked effects on the reproductive system that included reduced spermatogenesis and corpora lutea in male and female dogs, respectively (Yiannikouris and Jouany, 2002). In further studies with low level exposure to ZEA at 200µg BW for 7 days, a significant toxic effect was noted which included changes in the female reproductive tract. The dogs also showed reduction in serum antibodies and white blood cell levels with 25 and 50µg/kg/BW of ZEA (Yiannikouris and Jouany, 2002, Speijers and Speijers, 2004).

Although ruminants are not as sensitive to ZEA as swine, a few experiments have been done to determine whether ZEA affects the welfare of cattle. Infertility, reduced milk production,

and hyper-oestrogenism in cows have been reported in association with ZEA (Rocha et al., 2005, Gazzotti et al., 2015). Animals fed ZEA over three oestrous cycles developed poorer conception rates with up 25% reduction (Rocha et al., 2005). The potent oestrogenic effects of ZEA have been illustrated via laboratory animal studies in mice that were fed 30µg/kg of ZEA in the feed for 13 weeks which resulted in endometrial hyperplasia. The biological potency appears to be high, but toxicity low, as seen in female rats requiring a LD_{50} of 10,000 mg/kg/BW and female guinea pig about 5000mg/kg/BW (Yiannikouris and Jouany, 2002). Risk to humans was considered to be low and probably the reason for poor regulation as regards to its content in food. Nevertheless, the danger of a cocktail of mycotoxins in feed together with an immune compromised animal may result in the additive action and increased toxicity.

Permissible limits in animal feed is between 0.1 -0.5ppm depending on the production stage of particularly food animals with particular reference to pigs. Though the general limit in feed permitted is about 2-4ppm (EC Directive 2002).

Alternaria

Trichothecenes are a very large family of mycotoxins that are produced by myriad of *Fusarium* species as secondary metabolites. *Myrothecium sp*, *Trichoderma* sp *Trichoothecium sp* and *Stachybotrys* species of fungi have also been implicated in the production of trichothecenes (Sweeney, 1998). They are largely implicated in contamination of many different cereal grains like wheat, oats or maize but can also grow indoors on damp surfaces. They are ubiquitously found in the environment and are classified in to 4 groups (A-D) but groups A and B are most commonly cited in mycotoxicosis (Krska et al., 2001)Type A trichothecenes include HT-2 toxin and T-2 toxin. Type B trichothecenes type B includes deoxynivalenol (DON), nivalenol and fusarenon X. In the case of less prevalent C and D they include crotocin, baccharin, satratoxin and roridin. Type A is more relevant because of it implication in human and animal health (D'mello et al., 1999, Placinta et al., 1999).Some of clinical symptoms as result of ingestion are anorexia, vomiting and immuno-suppression (Fink-Gremmels, 1999). Limited recommendation as regards it limits in food and feed have been implemented.

Patulin (PAT) is a secondary metabolite produced by certain moulds of the genera *Penicillum* and *Aspergillus*. These fungi grow on fruit but maybe opportunistic on other food commodities. Patulin has been shown to exhibit toxic effects in animals (Anderson et al., 1979). It occurs most often in spoilt apples that may have mouldy growth and maybe seen in further processed

apple products. It has also been found opportunistically in other fruits, cereals and cheese but apples and apple products are the main substrate for patulin producing fungi. Majority of the information on the toxicity of patulin is extrapolated from laboratory animal studies and there is scanty information in humans. At high doses PAT is acutely toxic in mice and rats, causing gastrointestinal lesions including severe distension and haemorrhage in the upper gastrointestinal tract. At high doses PAT has resulted in cytotoxic changes (Hayes et al., 1979, McKinley et al, 1992). Not enough is known about its long term chronic exposure which necessitates further investigation.

Citrinin is a mycotoxin which is often found in various food cereals (Buckle, 1983). It is a secondary metabolite produced by *Penicillum species* of fungi but also known to be produced by *A. niveus* and *A. terreus*. Many animal studies have cited its cytotoxic and immunotoxic outcomes. (Kitchen et al., 1977, Reddy et al., 1988). Citrinin often occurs together with other mycotoxins like OTA or AFB_1, as they are produced by the same fungal species. Citrinin is most often seen in combination with OTA. This co-occurrence of these mycotoxins may result in either additive or combinative effects when present in a living organisms. The nephrotoxic effects of OTA and citrinin are additive and was evidently involved in the pathogenesis of a human kidney disease, called Balkan Endemic Nephropathy (Stoev, 2017). The strongest syngerstic effects are seen between OTA and penicillic acid, which are produced by the same fungi in practice, because of the impaired detoxification of OTA in the small intestine provoked by penicillic acid (Stoev et al 2001,2004).

Moniliformin is a contaminant of cereals which is produced by a number of *Fusarium* species that include *F. moniliforme*, *F. avenaceum*, *F. subglutinans*, *F. fujikuroi* and *F. proliferatum*, (Marasas et al., 1988b). Though moniliformin is produces by the Fusarium species, it has no similarity in structure to Fumonisin. It is mainly cardiotoxic and causes ventricular hypertrophy in a wide range of animals including rats, chickens and ducks (Ledoux et al., 1992) but a number of other potential diseases have been associated with it (Peltonen et al., 2010, Norred et al., 1996).

Cyclopiazonic acid is a secondary metabolite isolated from *Penicillium cyclopium* and subsequently from other fungi including *P. griseofulvum*, *P. camemberti*, *P. commune*, *A. flavus*, and *A. versicolor*e. Cyclopiazonic acid is chemically related to ergoline alkaloids and appear to be toxic in high concentrations in pigs and poultry(Lomax et al., 1984, Gentles et al., 1999).

The most prominent member of this ergot alkaloid group is *Claviceps purpurea* . This fungus grows on rye and related plants, producing alkaloids that can cause ergotism in mammals. The proliferation of fungal growth on the plant seed results in harden mass called sclerotia. This mass is a brown black ergot containing the ergot alkaloid when consumed, via the food chain, leads to illness. Ergotism is the name used for severe pathological syndromes affecting humans or other animals subsequent to the ingestion of plant material having sclerotia that contains an ergot alkaloids (Brook, 1963). "St Anthony's Fire" was the common name given to a condition by monks of the Hospital Brother of St. Anthony in 1095 (Zavaleta et al., 2001). This was in reference to severe burning sensations in the limbs as a result of consumption of contaminated grain. The effects of ergot alkaloids on the vascular system due to vasoconstriction with reduced blood supply results in gangrene and loss of limbs (Cross, 2003).

Mycotoxin extraction

Patterson and Roberts (1979), devised a multi-mycotoxin extraction method which is used for extractions of all the aflatoxins, zearalenone, and ochratoxin A. This methodology has become a standardised extraction process for most studies on mycotoxins that require extraction from feed. In order Acid (A) and neutral (N) fractions are obtained, extractions was done using aqueous acetonitrile containing potassium chloride and the toxins further extracted with dichloromethane with added sodium bicarbonate to obtain a neutral (N) fraction and after reacidification to obtain an acid (A) fraction. Except for ochratoxin A which is acidic, all other mycotoxins were in the neutral fractions. For fumonisin extraction method of Sydenham *et al.* (1992) and Shephard and Sewram (2004), with minor modifications are accepted practices.

Dog and Cat Immune System

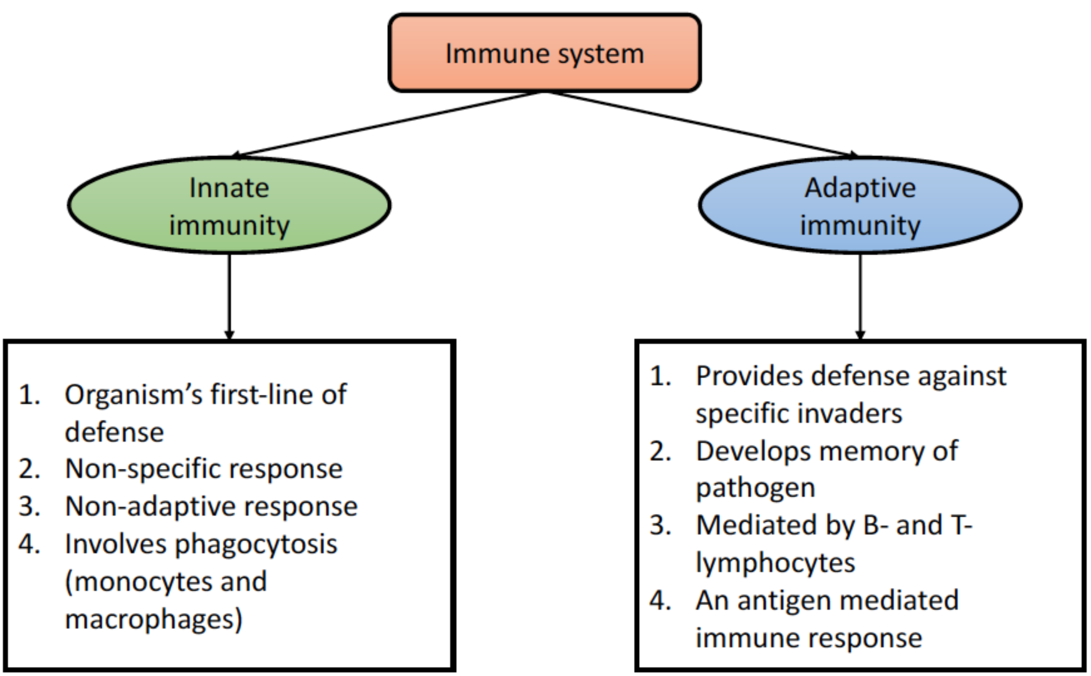

Figure 7. A basic summary of the functions of the different branches of the immune system (Prepared by author).

The immune system is the main defence against invading organisms and toxins. The key organs that support the immune system are thymus, liver and spleen. In poultry the bursa of Fabricus is most important. The complexity of the immune system is better evolved in mammals wherein various cells interact to elicit a solid and desired response to invasion by foreign bodies. The first line of defence is referred to as innate which is non-specific barrier to invasion or infection e.g., skin which is supported phagocytes, mucous and tissue chemicals that keep out foreign bodies. If the first line of defence is unsuccessful in arresting the invasion due foreign bodies or substance, the adaptive immune system become the next line of defence (Rock et al., 2011). Lymphocytes together with macrophages are major cellular units of the immune system. Lymphocytes differentiate into T cells and B cells. The T cell are activated when it binds to an antigen. These activated T cell will recognise any foreign bodies carrying the antigen and then attack and destroy it. In case of B cell, they recognise and attach to the antigen and activates a defence. The activation

supports production of antibodies or immuno-globulins that have a key role in destroying and mopping up the infection (Day, 2011).

Acute and Chronic Toxicity

Mycotoxins may gain access to both man and animals via ingestion, most commonly, but less commonly via derma, respiratory system and rarely parentally. Animals are commonly affected via direct ingestion of mouldy plant material generally as chronic exposure rather than acute exposure (Surai et al., 2008, Meissonnier et al., 2008). Acute toxicity would refer to short duration exposure wherein death occurs while chronic exposure would refer longer exposure to a toxic substance wherein the morbidity and mortality maybe high. Acute toxicity could result either from a single exposure or from multiple exposures in a short period of time. The prerequisite responses should occur within 14 days of the administration of the substance (Munday, 2006, Jonsson et al., 2013). Chronic toxicity is associated with long term exposure to a contaminant in excess of 14 days to a few months. Adverse effects associated with chronic toxicity can be directly lethal but are more commonly sub-lethal, which may include changes in growth, reproduction, or behaviour.

Many mycotoxins may elicit a acute (7 days) response rather than per-acute (24-48hrs) response seen often with AFB_1 that cause death within a week at dose of 0.5-1 mg/ AFB_1/kg body weight in dogs (Leung et al., 2006). Dogs exposed to lower doses of 0.05-0.3 mg/AFB_1 per kg body weight for 6-8 weeks would show the typical signs associated with acute phase of polydipsia, polyuria, lethargy, hepatitis and death. Jaundice is predominated symptom of chronic exposure (Stenske et al., 2006, Arnot et al., 2012a). Mycotoxins OTA, ZEN, FB_1 and FB_2 are generally associated with chronic exposure though many studies have not eluded clear time lines (Gajęcka et al., 2004, Adeneye, 2014). Dogs appeared more susceptible then cats to acute mycotoxicosis. Data on chronic exposure to is still unclear (Scudamore et al., 1997b).

Immune Disruptors

The role of mycotoxins in the humoral and cellular immune response is well described in the literature (Hueza et al., 2014, Al-Anati and Petzinger, 2006, Supriya et al., 2016). In

the case of deoxynivalenol (DON) it causes elevation of IgA and the concurrent depression of IgM and IgG seen in a mice trials with toxins mediated via feed. (Pestka, 2003). AFB_1 role as an immune disruptor is well published highlighting its absorption and concentration in liver (Krishnamachari et al., 1975, Greene et al., 1977, Dereszynski et al., 2008, Arnot et al., 2012b); it is metabolised via microsomal enzyme cytochrome P_{-450} 3A4 to various metabolites. These metabolites disrupt membrane integrity and result in cell lysis. In acute aflatoxicosis death results within a week (Azziz-Baumgartner et al., 2005) but chronic aflatoxicosis initiates immune suppression which is the preamble to cancer development (Frehse et al., 2015). The cellular immune response due to AF alludes to conflicting information in pigs (Oswald et al., 2003) but studies in mice and chickens are conclusive of its role as an immune disruptor (Ghosh et al., 1991, Dugyala and Sharma, 1996). OTA is a known carcinogen which tends to concentrate in blood and distributes to kidneys and liver. The effects of gene expression is regulated by nuclear factor –erythroid 2p45 related factors, hence having a profound effect on antioxidant production (Cavin et al., 2006). This leads to immunosuppression and induces apoptosis and DNA degradation, resulting in impairment of the vital immune organs, such as the thymus, spleen and lymph nodes, with a depression of antibody production (Seegers et al., 1994, Al-Anati and Petzinger, 2006). FB_1 and FB_2 are known to be cytotoxic with FB_1 known to traverse the placental barrier hence having a teratogenic nature (Stockmann-Juvala and Savolainen, 2008, Voss et al., 2007). FB_1 link's to disease is via increased oxidative DNA damage or alternatively via disruption of the de novo sphingolipid biosynthesis pathway (Merrill Jr et al., 2001). Inhibition of these pathways disrupts apoptosis and mitosis, which then contributes to carcinogenesis and cell death. The immunosuppressive effects of OTA on humoral and cellular responses is mainly due to the inhibition of protein synthesis (Creppy et al 1984) and to the competitive inhibition of intra-mitochondrial phosphate transport (Meisner and Chan, 1974) which subsequently leads to susceptibility to secondary infection in farm animals at 1 ppm. (Stoev et al 2000). The immunosuppression is the first toxic effect expressed by OTA. ZEA metabolites are less toxic then ZEA itself. The two metabolites alpha- and beta- zearalenol cause cell toxicity by damaging protein synthesis and DNA synthesis thereby inducing oxidative stress. ZEA and its derivatives compete for with 17 B-E2 specific binding sites on oestrogen receptor initiates a sequence of steps that results in oestrogenic stimulation (Kuiper-Goodman et al., 1987). Pathological outcomes often depend on the reproductive status of the animal (Etienne and Jemmali, 1982, Minervini and Dell'Aquila, 2008).

In general mycotoxins have an acute or chronic immune response that elicits cytotoxic and immunotoxic effects on DNA, RNA and protein synthesis resulting in changes in normal cellular function of growth and development.

Challenges -Regulation and Quality control

The demands on food and feed security for man and animal, respectively, will become an increasingly global challenge. Climate changes will affect agricultural practices and production, primarily challenging supply and demand, and consequently how animal feed is formulated. The drive for the least cost feed formulation will result in substitution of feed ingredients with sub-standard material rejected from human food production. Many of these substandard material will find their way into the animal food chain. Hence the future of the animal feed industry, notwithstanding human food too, lies in clear regulation and specified feed formulation. In specific reference to mycotoxins, mycotoxin limits need to be clearly defined for, especially, major toxins implicated in mycotoxicosis. In addition, there needs to be a global standard for feed ingredients and permissible limits in finished products. The quality control concept of HACCP which is widely used in the human food sector needs to be implemented wherein greater attention is paid to manufacturing inputs and feed ingredients. (Panisello et al., 2000). In the human food retail sector labelling of products are clear with regards to nutrients and bioavailability. Many reputable retail outlets require that suppliers adhere to strict standards that often exceed the governmental regulation or stipulation. This maybe a cue for the animal feed sector. The pet food industry needs to review it entire quality control system to ensure standards and safety by implementation HACCP quality control system combined with responsibility by manufacturers to exceed standards set by regulation bodies.

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Chapter 3 - Paper 1

A comparative analysis of mycotoxin contamination of supermarket and premium brand pelleted dog food in Durban, South Africa

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Abstract

Dry pelleted dog food in the South African market is available via supermarket, pet stores (standard brands - SB) and veterinary channels (premium brands-PB). For the purpose of this study, the supermarket channel included the cheaper quality foods and premium brands were sold via the veterinary channel (N=20). These feeds were analysed for four main mycotoxins viz. aflatoxins (AF), fumonisin (FB), ochratoxin A (OTA), and zearalenone (ZEA) using standard well-described extraction, characterisation and quantitation processes. Irrespective of the brand or marketing channel all foods were contaminated with fungi (mainly *Aspergillus flavus, Aspergillus fumigatus* and *Aspergillus parasiticus)* and mycotoxins (most prevalent being aflatoxins and fumonisins). This was observed in all 20 samples irrespective of the marketing channel or perceived quality. Also, many samples within each marketing channel failed the 10 ppb limit for aflatoxin set by regulations in South Africa. Though fumonisin was detected in all samples, a single sample, failed the Food and Drug Administration (FDA) limit of 10,000 ppb. Both OTA and ZEA was found at low levels and was absent in some samples. This study shows that higher priced dog food does not ensure superior quality or that it is free from contamination of either fungus or mycotoxins. However, the more expensive PBs did show contamination levels that were lower than the cheaper SBs.

Key words: Mycotoxins, fungi, aflatoxins, fumonisins, ochratoxin A, zearalenone, marketing channels, standard and premium brands

Introduction

A global trend of increasing pet ownership, with the concurrent affluence of modern society, has led to a greater demand for specialized pet products and diets. The manner that pets are fed is influenced by societal habits and income, as a result of this market trend many new products are developed yearly (Pet Food Pet Market., 2016). Most pet foods are adequately labelled as regards their ingredients and nutritional content. Hence, many pet food brands are registered and accredited with some consumer interest group, such as the Pet Food Institute (PFI), Association of American Feed Control Officials (AAFCO) or a governmental organisation as in South Africa in terms of the Fertilizer, Farm Feeds, Agricultural Remedies and Stock Remedies (Act No.36 of 1947; South African Government, 2009) or similar depending on its geographical location. Dry pelleted pet food often contains 5-28 % of animal protein, or its derivatives with the remaining portion consisting of maize, maize gluten, wheat, wheat gluten, rice and its by-products amongst other "millings" (Klich and Pitt, 1988, Brown, 1997). However, none of these account for the digestibility of the diets or content of chemical agents and potential toxins. The remaining portion consists of vegetable matter, bulking agents and chemical additives. In a highly competitive and price driven pet food market the use of inferior maize, maize gluten, wheat, wheat gluten, rice and its by-products are common. In addition, inferior quality slaughterhouse renderings and milling by-products make up the fillers in the formulation of price driven pet diets (Klich and Pitt, 1988).

Examination of dog food packaging and product labels, indicate that claims of high crude protein content is largely vegetable in nature and animal by-products which are minimally from meat sources. Packaging labels provide extensive information regarding ingredients, however with limited information on actual percentages of ingredients used in the formulation. This trend has been observed in both Premium brands (PB) and Supermarket brands (SB), market segmentation often leads to misunderstanding amongst consumers as regards to the nutritional value of the product. PB often percentages of protein from fresh meat sources. Cereal products that are considered unfit for human consumption are often incorporated into feed formulations and act as excellent substrates for microorganisms such as fungi to proliferate. These contaminated cereals (Tulpule, 1981, Bennett and Klich, 2003) often become a health risk to pets resulting in mycotoxicosis outbreaks with associated morbidity and mortality. The cheaper SB and "home industry" preparations have often been implicated in mycotoxin poisoning in domestic animals and pets which resulted in severe clinical symptoms that included depression,

anorexia, and weakness with sudden death. In the case of chronic mycotoxin exposure, all the acute symptoms prevailed, except sudden death, with lingering weight loss, jaundice and organ failure (Dereszynski et al., 2008, Newman et al., 2007).

We analysed dry pelleted dog food (both PB and SB) for some of major pathogenic fungi and common mycotoxins. Foods purchased included premium brands (PB –veterinary: higher price ranges R40.00-R100.00/kg) and SB (supermarket: low price range – R 4.00- R30.00/kg) sold in Durban, South Africa. PB are those brands that are exclusively sold via veterinary practices and retail veterinary shops, while SB were exclusively purchased at supermarkets and generally not endorsed by veterinarians. PB feeds are perceived to contain more protein from meat sources but, in reality, are not devoid of cereals and meat by-products which also make them susceptible to mycotoxin contamination. SB contain cereals and meat by-products as major ingredients. Many of the brands have many life stage variants within its product range, hence special care was taken not to repeat brands while sampling. Therefore, we kept most of the choices to the adult range variant for consistency. The general perception that the expensive PB sold through veterinary clinics and specialized veterinary outlets are the best quality was challenged when compared to the SB. These samples were evaluted for the amounts of aflatoxins (B_1 and B_2), fumonisins (B_1 and B_2), OTA and ZEA in both marketing categories. Veterinary knowledge on the potential range of toxicological effects is still limited, though its serious health effects has been recorded and scribed (Bryden, 2012, Boermans and Leung, 2007). The mycotoxins investigated have been most commonly implicated in mycotoxin outbreaks associated with domesticated pets and livestock. This study aimed to identify, quantify and compare fungal and some commonly found mycotoxin profiles for both marketing channels under recommended legislation. This study further provides information to both veterinarians and consumers as regards to making informed choices in their preference of purchase of their pet food. These choices have a direct impact on the pet's long-term health and welfare.

Materials and Methods

Materials

All chemicals, reagents and mycotoxin standards were obtained from Merck (South Africa) and Sigma (South Africa) unless otherwise specified. A total of 20 dog dry pelleted food brands were purchased, all samples were purchased via the respective marketing channel (10 per marketing channel) and catalogued as brand name and serial and batch numbers. All samples were well within the expiry date listed on each pack. All mycotoxin standards were obtained from Sigma (St. Louis, USA). FB_1 and FB_2 were purchased from PROMEC (MRC, South Africa).

Methodology

Sampling

All 20 bags (10 PB and 10 SB) of pelleted dog food were opened and mixed prior to obtaining a representative sample of 500g. This representative sample was placed and sealed in a new clean plastic bag. These samples were then prepared for fungal and mycotoxin analysis.

Sample preparations

The sealed bags (500g pelleted dog food) were well shaken, opened and a 200g sample was weighed and placed in a blender jar. All feed samples were then milled to a fine powder using a mechanical blender (Petron 3600, Germany). The milled samples were used for fungal analysis and mycotoxin determination. Remaining samples were resealed and stored in individual sealed containers at 4^oC until required for further analysis.

Fungal Isolation

Fungal isolation was done by pipetting 1 ml of serially diluted 1g blended material suspended in 9ml Ringer's solution on potato dextrose agar (PDA) and Ohio Agricultural Experimental Station agar (OAESA) (Kaufman et al., 1963); sub-culturing of isolated colonies on PDA, malt extract agar (MEA) and Czapek yeast extract agar (CYA) followed by macro- and microscopic

identification. Determination of each species of fungi was done using the keys of Klich and Pitt (1988) and Klich (2002) for *Aspergillus* spp. and Pitt and Hocking (1997) for *Penicillium* and other genera. This was done by observing both macroscopic characteristics of the colonies on various media used as well as the microscopic morphology and measurements of the conidiophores (after staining mycelia with 0.1% fuchsin dissolved in lactic acid) under an Olympus B061 Compound microscope (Wirsam Scientific, South Africa) and Microscope Standard 19 (470919-9902/06), equipped with an Axiocam MRC Camera Ser. No. 2 08 06 0245 and AxioVision Release 4.5 SP1 (03/2006) software (Zeiss, West Germany).

Mycotoxin extraction and clean-up of feed samples

A multi-mycotoxin extraction method (multi-mycotoxin screen) devised by (Patterson and Roberts, 1979) was used for extraction of all the AFs, ZEA, and OTA. Briefly, 25g of milled dog food was extracted using aqueous acetonitrile containing potassium chloride and the toxins further extracted with dichloromethane with added sodium bicarbonate to obtain a neutral (N) fraction and after reacidification to obtain an acid (A) fraction. The N fraction was dialysed against 30% aqueous acetone overnight and then back-extracted into dichloromethane. The two fractions were evaporated and dried under a nitrogen gas stream and stored in sealed vials. Except for OTA that was in the A fraction, all other mycotoxins of interest were in the N fraction.

For fumonisins, the extraction and clean-up was done according to the method of Shephard and Sewram (Shephard and Sewram, 2004) with minor modifications. Milled pelleted food sample (25g) was extracted with 50ml of methanol: water (3:1) and after shaking on a bench shaker (1hr), the entire content was filtered through a Whatman no. 2V filter paper. The filtrate was passed through a previously conditioned strong anion cartridge (SAX) column (Bond Elute, VARIAN, South Africa) with 5ml methanol followed by 5ml methanol: water (3:1 v/v). The column was washed with 8ml methanol: water (3:1, v/v) and then 3ml methanol. The absorbed fumonisins were then eluted with 10ml 1% acetic acid in methanol. The eluent was evaporated and dried under nitrogen gas and the residue stored in a screw-cap vial (4°C) until analysed.

For confirmation, aflatoxins (AFB_1, AFB_2) were extracted using an immuno-affinity column (VICAM) using the VICAM method as follow: to milled feed (25g), 5g NaCl was added to samples and mixed with 100ml of methanol/water (80/20; v/v) and blended for 1min. The

extract was filtered successively through fluted filter paper and 10ml diluted to 50ml with water, mixed and filtered using a microfiber filter. Finally, 10ml of the final filtered extract was passed through the immuno-affinity column followed by 10x 2ml of distilled water. Aflatoxins were eluted with 1ml methanol, dried under nitrogen gas and stored until analysis.

Thin layer chromatography (TLC)

To detect each mycotoxin of interest, two-dimensional TLC was performed (Patterson and Roberts, 1979). Briefly, into the vial containing mycotoxin extract, 20µl of 200 µl DCM-containing extract solution was spotted on a 10 x 10mm silica gel TLC plate and a two-dimensional TLC performed using appropriate mobile phases. After the mobile phase reached the top of the plate, the plate was air dried and viewed under UV light (for fluorescent detection spots) or treated with P-anisaldehyde and heated in the oven for a minute for fumonisin detection.

High Performance Liquid Chromatographic analysis of feed sample extracts (HPLC)

Aflatoxins B_1 and B_2, ZEA, OTA and fumonisins (B_1 and B_2) were quantified in the appropriate fractions of the sample extracts by HPLC. The mycotoxin extracts were dissolved in 1ml methanol and filtered through a 0.2 µm Millipore filter; the final filtrate was used as the analyte. The chromatographic separation of analytes and standards was performed by passing through the Symmetry column with an operational oven temperature of 30°C.

AFB_1 and AFB_2 were individually determined using HPLC with fluorescence detection after post-column electrochemical derivatization with bromine using a KOBRA cell (Chu, 1991). The eluent was water-methanol (58:42, v/v) with the addition of 119mg potassium bromide and 100µl nitric acids (65%) per litre at an isocratic flow-rate of 0.8ml/min. The aflatoxins were detected using a scanning fluorescence detector at excitation and emission wavelengths of (360nm, 440nm). ZEA was analysed by fluorescent detection at excitation and emission wavelengths of 274nm and 418nm respectively. The injection volume was set at 20µl while the mobile phase (acetonitrile/water (45:55 v/v) was pumped at the rate of 1ml/min. OTA analysis was measured using fluorescence detection (Allcroft et al., 1961). The mobile phase consisted of acetonitrile: water: acetic acid (50:48:2 v/v/v) was pumped at a rate of 1ml/min. Respective fluorescence excitation and emission wavelengths of 334nm and 460nm were used.

Fumonisin containing extracts were reconstituted in methanol, and 50µl aliquots derivatized with 250µl of *o*-pthaldialdehyde (OPA), prior to separation on a reversed-phase HPLC system using fluorescence detection at excitation and emission wavelengths of 335nm and 440nm respectively (Shephard and Sewram, 2004). The isocratic mobile phase made up of 0.1M dehydrated sodium dihydrogen orthophosphate: methanol (80:20) with pH adjusted to 3.5 using orthophosphoric acid, was pumped at a rate of 1ml/min. The injection volume was 50µl.

For recovery, selected feed samples with known concentrations were spiked with 100µg/kg of AFB_1, AFB_2, OTA, ZEA and 200µg/kg of FB_1 and FB_2 for determination of recoveries. The mean recoveries obtained in triplicate were respectively 98.2% and 96.5 % for $FB_1$and FB_2 and for AFB_1 and AFB_2, was 95.5% and 89 % respectively, while ZEA and OTA recoveries were 93.0 and 94.6%, respectively (Table 1).

All samples were analysed on Shimadzu Corporation (Kyoto, Japan) LC-20AB liquid chromatograph equipped with CBM-20A communication bus module, LC-20AB degasser, CTO-20A column oven, Nova-Pak 4mm C18 reversed phase analytical column (250×4.6 mm, 5µm), SIL-20A auto sampler, RF-10AxL fluorescence detector, RID-10A refractive index detector and SPD-M20A photodiode array detector linked to LC solutions version 1.22 Software Release.

Results

The 20 dog food samples (10 PB and 10 SB) were cultured for fungal isolates and reported as colony forming units per ml (CFU's/ml) (Table 2) and mycotoxin contamination using TLC and HPLC. First TLC characterisation was performed to detect the four most commonly occurring mycotoxins viz., AFB_1 and B_2, FB_1 and FB_2, OTA and ZEA. Thereafter, the feed extracts containing these mycotoxins were quantified using HPLC (µg/ml) (Table 1).

Table 1. HPLC recovery of the selected mycotoxins after spiking with appropriate amounts of the pure standard.

Mycotoxin	Concentration spiked (µg/kg)	Concentration measured (µg/kg)	% Recovery
AFB_1	100	95.5	95.5
AFB_2	100	89.0	89.0
OTA	100	94.6	94.6
ZEA	100	93.0	93.0
FB_1	200	196.4	98.2
FB_2	200	193.0	96.5

The most prevalent fungal isolates in all dog samples were *Aspergillus* species, *Fusarium* species and *Penicillium* species amongst others (Table 2). These fungal species were found in both PB and SB categories. The fungal isolates of *Aspergillus flavus*, *Aspergillus fumigatus* and *Aspergillus parasiticus* were the most prevalent in both categories but the PB showed highest levels of *A. flavus* (500 x 10^4 CFU's) as compared to SB. The other fungal occurrences of *Aspergillus species* were similar for both categories. The SB had a higher number of fungal isolates for *Fusarium graminerium* while *Fusarium verticilloides* was similar for both categories. Isolates of *Penicillum* were similar for both PB and SB dog feeds. In general, there was a prevalence of a variety of fungal species in both feed categories with *Aspergillus species* being the most abundant that is commonly implicated in aflatoxicosis.

The rapid TLC method allows for quick identification of the mycotoxins present in the feed extracts (using Rf values and spiking with known standards). The PB and SB samples displayed the presence of all four mycotoxins evaluated. PB showed higher levels of FB and lower levels for the other three mycotoxins while SB displayed moderate levels of AF and FB with low levels of OTA and ZEA.

All 20 samples were contaminated with AF. Both PB (20.17µg/kg) and SB (44.17µg/kg) showed high levels AF's with AFB_1 exceeding the levels set for AF's of 10 ppb limit regulated by the Fertilizer, Farm Feeds, Agricultural Remedies and Stock remedies Act No.36 of 1947

(South African Government, 2009) and similar international standards of the (FDA, 2001). Five PB and five SB samples failed the 10ppb limit. Two SB samples exceeded 100 ppb for fumonisin, OTA and ZEA were detected, by HPLC, in most samples at very low levels. ZEA is produced by *F. graminerium, F. nivale,* and *F. avenaceum,* and has been implicated in reproductive pathology of canines. OTA, a nephrotoxin, is produced by a number of *Aspergillus* and *Penicillium* species.

Discussion

Companion animal ownership has evolved from being a peripheral addition to an integral part of modern human life. In the USA, the total cat and dog ownership is about 150 million and is worth $50 billion and this translates to about 65% of households owning at least one pet, with pet ownership expected to grow by 4-5% each year (Pet Care Analysis, 2017). The trend of humanisation has driven the pet food market forward as humans seek higher quality foods and accessories for their companion animals, to improve the quality of life for their pet(s). Companion animals have moved from being "outside pets" to be members of the inner circle of human daily household life. The drive for convenient food products amongst consumers extends to their pets hence increasing animal welfare awareness. The consumer would rather buy conveniently packed pelleted or canned food for the pet than the raw ingredients (Pet Food Market, 2016). The perception of higher-priced pet foods found in the PB channel being of better quality was investigated. The analysis of dry pelleted food for major pathogenic fungi and mycotoxins in both marketing channels allowed us to compare and test this hypothesis.

Pet foods (cheaper and low quality sold via supermarkets and "home industry") are known to contain more cereals and cereal by-products – leading to the common assumption that these would be heavily contaminated with fungi as compared to the more expensive food brands. This ideology was found to be questionable based on the analysis of our 20 samples tested in both PB and SB marketing channels. This was of particular concern since both *A. flavus* and *A. parasiticus* were detected at high levels (300-500 x 10^4 CFU's). These fungi were previously implicated in AF outbreaks in domestic animals (Arnot et al., 2012, Fox et al., 2012). These fungi commonly produce AF that are potent hepatotoxins and hepatocarcinogens that may lead to serious clinical symptoms such as depression, loss of appetite, weight loss and sometimes sudden death (Newman et al., 2007, Dereszynski et al., 2008, Fox et al., 2012). Dogs'

susceptibility to AF's is attributed to their low glutathione S transferase activity that plays an important role in detoxification of this mycotoxin (Dereszynski et al., 2008). In many AF outbreaks dogs were found to be susceptible to low dose ranges of 50-300µg/kg for 42-48 days and high doses of 500-1 000µg/kg BW for acute cases (Lazicka and Orzechowski, 2010). AFB_1 is highly toxic as compared to its other forms and has been the main aetiological cause of dog deaths at dose levels of 223-579µg/kg food resulting in severe liver failure (Krishnamachari et al., 1975, Newman et al., 2007). In our study, these levels often exceed tolerable levels for canines and are implicated in many canine aflatoxicosis (Newman et al., 2007, Arnot et al., 2012) (Table 1).

Our findings are not surprising as these are ubiquitous soil fungi that contaminate agricultural crops like maize, groundnuts and other cereal grains (Leung et al., 2006) and are implicated in mycotoxicosis. The SB had a higher number of fungal isolates for *Fusarium graminerium* while *Fusarium verticilloides* was similar for both categories (Table 2). Fusarium mycotoxins are a broad and diverse group that have been implicated in a wide variety of clinical symptoms in animal toxicology (Placinta et al., 1999). Aspergillus and Penicillium species are known to be implicated in ochratoxicosis, caused by the nephrotoxin OTA (Leung et al., 2006), Recent investigations have lent support to a multi-aetiological syndrome with regards to mycotoxin poisonings. Publications emanating from South Africa (Arnot et al., 2012) and Israel (Fox et al., 2012) together with analytical work around pet foods and cereal ingredients (Fox et al., 2012, Mwanza et al., 2013) substantially supports this idea.

Table 2. Fungal identification and their approximate quantitation (colony forming units: CFU) in PB and SB feeds after culture

Fungal isolates	Genus	Species	*Premium*	*Standard*
	Aspergillus	*A. flavus*	***	**
		A. fumigatus	*	**
		A. niger	*	*
		A. niveus	*	-
		A. ochraceus	*	-
		A. parasiticus	**	**
		A. penicilioides	-	*
		A. poae	-	-
	Fusarium	*F. graminerium*	**	***
		F. verticilloides	*	*
	Penicillium	*Penicillium spp.*	**	**
		P. polonicum	-	-
		P. crostosum	-	-
	Other	*Rhizopus spp.*	+	-
		Unidentified microbe	-	-
		Yeast	+	+

KEY	
*	100 – 300 x 10^4 CFU's
**	300 – 500 x 10^4 CFU's
***	>500 x 10^4 CFU's
+	Positive only

The case of fumonisin and human disease seems to have poor correlation, this is however, not true for animals. Feeds contaminated with *F. verticilloides* (produce fumonisins especially FB_1) have shown cardiotoxicity and cardio-respiratory symptoms in pigs (Harrison et al.,

1990). The *Fusarium sp.* toxins, viz., fumonisin, zearalenone and trichothecenes are all implicated in adverse animal health (Placinta et al., 1999). At this point not much information is available as regards either FB_1 or FB_2 implication in dog toxicity and further their prescribed minimum levels in pet foods is unclear. However, their contribution to animal ill health and immunosuppression cannot be ignored (Boermans and Leung, 2007, D'mello et al., 1999); their contribution to serious disease in other species such as equines (leukoencephalomalacia), swine (hepatitis and pulmonary oedema) and rodents (hepatic and renal) was cited by Voss et al. (2007), (Placinta et al., 1999). FB's were detected in all of samples of PB and SB by HPLC (Table 1). A single SB sample failed the 10,000 ppb limit set by the FDA for animal feeds. The FDA (2001) sets its limits between 1000 ppb for equines up to 50 000 ppb for poultry whilst Canada, Japan and many countries sets no limits for fumonisins in pet foods (FAO, 2004)

OTA is nephrotoxic in companion animals and produces a wide range of symptoms varying from anorexia, weight loss, dehydration, and haemorrhage (Szczech et al., 1973a). A study on beagle dogs showed susceptibility and vulnerability to both acute exposure of 7.8mg/kg BW and chronic exposure of 200µg/kg BW resulted in anorexia, dehydration, gastric symptoms, prostration and eventual death (Szczech et al., 1973b, Razzazi et al., 2001).

OTA levels in both feed categories (PB and SB) was found to be at very low levels (Figure 1). Our study concurred with a similar study in Europe where OTA, though present in mycotoxin mix was found at much lower levels as compared to AF's and FB's (Gajęcka et al., 2004). OTA, a nephrotoxin, is produced by a number of *Aspergillus* and *Penicillium* species (Leung et al., 2006, Shephard and Sewram, 2004) displayed a similar quantitation trend as ZEA. Other studies (Gajęcka et al., 2004, Lazicka and Orzechowski, 2010) found that OTA and ZEA was less prevalent then AF's and FB's in their analysis similar to our study.

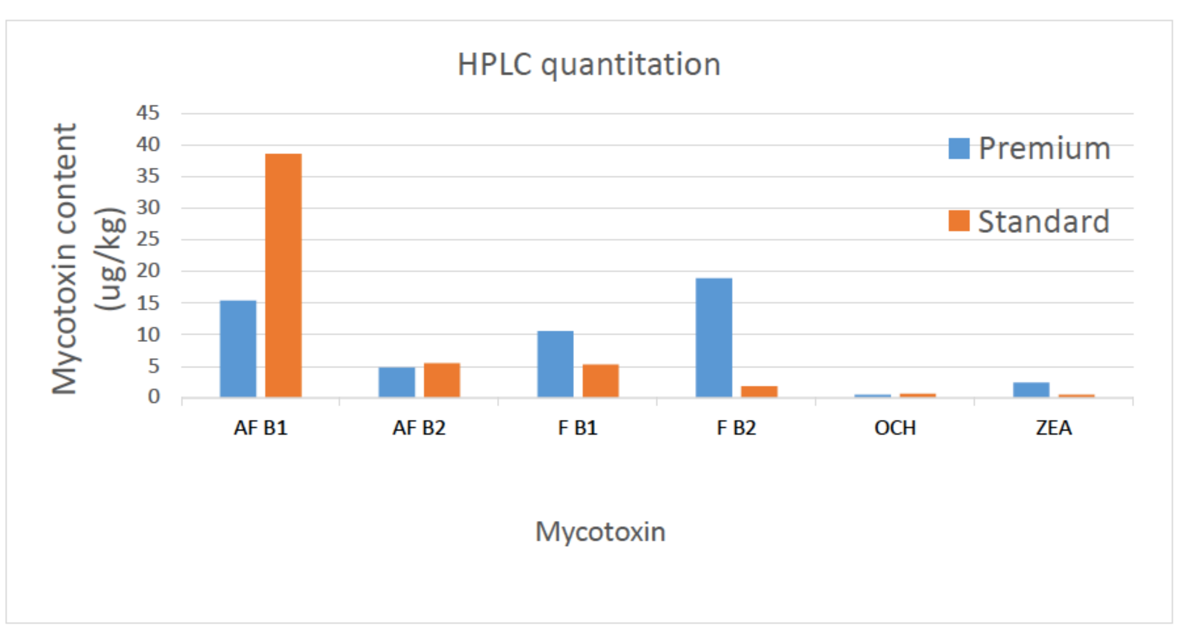

Figure 1. HPLC quantitation of the four major mycotoxins investigated in this study. Data is represented as a mean of two runs (With duplicate runs, no statistical analysis was possible).

ZEA detection by HPLC was limited and when detected was present in very low levels (2.4µg/kg in PB and 0.5µg/kg in SB) (Table 1). ZEA, a product of *F. graminerium*, *F. nivale*, and *F. avenaceum*, has been implicated in reproductive pathology of canines (Rotini et al., 2016). A study by Lazicka and Orzechowski (2010) found significant contamination of ZEA with a high of 298µg/kg parallels other studies in the Europe. ZEA toxicity correlated very closely with diseases of the reproductive system after exposure to 200µg/kg BW of toxin for a week (Gajęcka et al., 2004) .

Irrespective of the marketing channel or price range, all samples tested positive for AFB_1, AFB_2, FB_1 and or FB_2 notwithstanding the presence of OTA and ZEA amongst many samples. All foods tested presented a potential health risk to dogs. This observation then begs the question: does price ensure a mycotoxin free pet food? Do PBs ensure better and higher quality ingredients and quality control? Producers of superior brands of dog food claim that their ingredients used in the formulation of pet food are superior. The labelling requirement by both

governmental and non-governmental groups requires that the nutritional content and ingredients used in formulation be listed on the packaging. But this claim does not ensure mycotoxin free ingredients which remain an area of risk for the consumer and the pet (Fox et al., 2012). However, an important influencing factor for fungal growth and mycotoxin production is directly related to how the product is handled and stored post-harvest and manufactured in the case of pelleted dog food (Tulpule, 1981, Bryden, 2012). In South Africa, PB dog foods are imported (from the United States of America or Europe) and transported to SA by ship. It could be that this mode of transport may present an opportunity for the proliferation of fungi (the holding facilities may be damp and not well aerated) and their subsequent production of mycotoxins whilst in a container with high humidity and extremes of heat (Maia and Pereira Bastos de Siqueira, 2002). The problem may be exacerbated by the high levels of nutrients in PB food that could provide an ideal substrate for the production of mycotoxins (Gourama and Bullerman, 1995). This may explain the high levels of AF's in PB foods that exceeded the minimum limits prescribed (10ppb). SB products are often price driven in a highly competitive market and it is understandable that poor quality ingredients are often used in its formulation. The significant levels of AF's and FB's in both PB and SB presents a potential risk particularly with regard to the mycotoxin mix present in dog foods globally.

Conclusion

Our study shows that dog foods purchased based on price and marketing channels does not ensure a mycotoxin free product. It was surprising that PBs, in some cases, contained a higher mycotoxin content than SBs. Some SB brands did, however have a few samples that exceeded the prescribed limit up to 10 times the limit set by the Fertilizer, Farm Feeds, Agricultural Remedies and Stock Remedies Act No.36 of 1947 (South African Government, 2009). With a booming pet industry, opportunistic investors are always looking for quick return on investments. This subjects the pet food industry to economic predators that compromise quality for huge profit. This gives rise to the question of quality control in both procurement, feed formulation and production that requires further research and investigation. Concise labelling presents a challenge with present practice providing limited and often nebulous information. Label words such as "derivatives" and "by products" are vague and do not reflect the true content of the feed formulation. With respect to mycotoxins, the ingredients should clearly state the quantity of cereals present with an indication of approximate levels of the most

commonly occurring mycotoxins. Clear rules of engagement should be provided for storage of feeds (open and closed bags) and the implementation of HACCP principles in feed manufacture will improve the quality of the end product (Horchner and Pointon, 2011). Further studies into the use of AF-free transgenic maize (Thakare et al, 2017) together with improved processing and packaging technology may provide a possible answer to our questions. These are serious ethical considerations surrounding animal welfare and food safety to companion animals.

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Chapter 4 - Paper 2

A comparison of mycotoxin contamination of premium and grocery brands of pelleted cat food in South Africa

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Abstract

Contamination with mycotoxins is of concern to pet owners and veterinary practitioners due to their ability to cause disease and exacerbate the pathological changes associated with other diseases. Currently, there is a lack of information regarding the mycotoxin content of common premium (PB) and grocery brand (GB) cat feeds. Therefore, we undertook to determine the mycobiota content of feed samples, from both categories (n=6 each), and measured the levels of aflatoxin (AF), fumonisin (FB), ochratoxin (OTA) and zearalenone (ZEA) by HPLC analysis. There were high concentrations of mycotoxins in both categories of feed, regardless of the notion that premium brands are of a higher quality. The concentration of these toxins may contribute to the development of related pathologies in felines.

Key words: Premium brands, grocery brands, aflatoxin, fumonisin, ochratoxin and zearalenone

Introduction

Mycotoxins have been implicated in adverse effects in both human and animal health (Fink-Gremmels 1999; Pulina et al. 2014). In a worldwide survey (2004-2011) of over 17,000 samples of feed or feed ingredients it was found that more than 75% of samples were contaminated by at least one mycotoxin and 40% of the samples contained at least two mycotoxins (Streit et al. 2013). Currently about 300 mycotoxins have been identified but not all are necessarily implicated in toxicity. The Food and Agriculture Organization (FAO) estimates that a quarter of the food produced globally is contaminated with mycotoxins. This causes significant economic losses as well as posing a serious threat to human and animal health (Bryden 2012; Vasanthi & Bhat 1998). Hence, regulatory limits have been recommended by organisations such as the Food and Drug Administration (FDA) for the common mycotoxins. Mycotoxins commonly implicated in and associated with animal health concerns include aflatoxin (AF), fumonisin (FB), ochratoxin A (OTA), trichothecenes and zearalenone (ZEA) (Boermans & Leung 2007).

Dry pelleted pet food often contains 5-25 % of animal protein or its derivatives with the remaining ingredients consisting of corn, corn gluten, wheat, wheat gluten, and rice and its by-products, amongst other “millings” (Klich & Pitt 1988). In a highly competitive pet food market cost-cutting exercises are inevitable, leading to a compromise in the quality of products entering the retail sector. These cereal products that are often unfit for human consumption can act as excellent substrates for fungal proliferation and production of mycotoxins that contribute to liver, kidney and other diseases in pets (Bucci et al. 1998; Dereszynski et al. 2008). It is the contamination of cereals at harvest, post-harvest, manufacture and then storage (Bennett & Klich 2003; Tulpule 1981) that often becomes a health risk to pets by causing mycotoxicosis incidents and often death. In 2011, South Africa experienced an outbreak of aflatoxicosis as a result of the consumption of poor quality, low cost pelleted food (Arnot et al. 2012).The exacerbating factor was mouldy and low grade peanuts that were contaminated with *Aspergillus flavus* and *Aspergillus parasiticus*.

In this study, we compared the mycotoxin profiles of premium (PB) and grocery brands (GB) of cat food. PB products are perceived to have low amounts of cereal whilst GB are perceived to have a higher cereal content. Though no major mycotoxin outbreaks have been recorded in cats in recent years, the implication of mycotoxins and its role in feline health cannot be

excluded (de Souza & Scussel 2012). Examination of cat food labelling on packaging reveals that claims of high crude protein contents refer largely to vegetable and animal by-products and minimally to meat. Packaging labels provide extensive information on ingredients but limited information on actual percentages of ingredients in the formulation, a trend that is seen in both market segments and often leads to misunderstanding amongst consumers with regard to the nutritional value of the product. Furthermore, information gained from this study may warrant further investigation and provide a contribution to consumer knowledge and feline health.

Materials and Methods

Materials

Chemicals, reagents and mycotoxin standards were obtained from Merck (South Africa) and Sigma (South Africa) unless otherwise specified. All mycotoxin standards except Fumonisin B_1 (FB_1) and FB_2 were obtained from Sigma (St. Louis, USA). Fumonisin B_1 (FB_1) and FB_2 were purchased from PROMEC (MRC, South Africa). For this study PB refers to all veterinary restricted brands that may be purchased at veterinary practices or retail veterinary shops (Vetshops) only and generally are priced in the range between R80.00-R120.00 per kilogram, whilst GB are commonly sold in supermarket and grocery outlets at lower price range of R30-R60 per kilogram.

Methodology

Sampling

Pelleted cat food (n=12) from two marketing channels (PB and GB) were selected for this study. Samples were purchased from their respective outlets in convenient sizes of 2 - 3 kilogram packets. Information on brand, package size, and expiry date and barcode serial numbers were recorded. Each packet of food was emptied into a 5-litre bucket and thoroughly mixed by shaking. The sampling technique was adapted from methods described by Tittlemier *et al*. (2011). The bucket was divided into quadrants and approximately 125 gm per quadrant sample was scooped up with a clean metal ladle. The samples were thoroughly mixed prior to obtaining a representative sub sample of 500g of which a further sub sample of 200g was taken

by dividing 500g into 4 sub samples and 50 g taken from each quadrant. All feed samples (200g each) were milled to a fine powder using a mechanical blender (Petron 3600, Germany). The milled samples were used for fungal culture and mycotoxin determination. Remaining samples were resealed and stored in sealed containers at 4°C until required for further analysis.

Fungal isolation

Fungal isolation as well as sub-culturing and subsequent identification of fungi was done as previously described (Kaufman, Williams & Sumner 1963; Singh & Chuturgoon 2017).

Mycotoxin extraction and clean-up of feed samples

Mycotoxin extractions were done as described (Singh & Chuturgoon 2017).

Thin layer chromatography (TLC)

TLC was run for each mycotoxin as described (Singh & Chuturgoon 2017).

High Performance Liquid Chromatographic (HPLC) analysis of feed sample extracts

HPLC analysis of feed sample extracts was performed as previously described (Singh & Chuturgoon 2017).

Results

TLC characterisation and HPLC quantitation (µg/ml) were performed for the commonly suspected mycotoxins implicated in pet food contamination viz. AF, FB, OTA and ZEA (Liggett *et al*. 1986; Shephard & Sewram 2004; Stenske *et al*. 2006). The most prevalent fungal isolates in all samples were *Aspergillus* species, *Fusarium* species and *Penicillum* species (Table 2). These fungi were found in both PB and GB feed categories. The fungal species *Aspergillus flavus*, *A. fumigatus* and *A. niger* were more commonly isolated while *A. parasiticus, A. ochraceus, A. poae and A. penicilloides* were found less commonly in the samples tested.

Table 1: Mean recoveries of selected mycotoxins after spiking in feed samples using HPLC

Mycotoxin	Concentration spiked (µg/kg)	Concentration measured (µg/kg)	% Recovery
AFB_1	100	95.5	95.5
AFB_2	100	89.0	89.0
OTA	100	94.6	94.6
ZEA	100	93.0	93.0
FB_1	200	196.4	98.2
FB_2	200	193.0	96.5

Table 2: Fungal species identification and selected mycotoxin detection in premium and grocery brand cat pelleted feed samples

			Premium	***Grocery***
Fungal isolates (CFU's/mL)	*Aspergillus*	*A. flavus*	***	**
		A. fumigatus	*	**
		A. niger	**	**
		A. niveus	-	-
		A. ochraceus	-	**
		A. parasiticus	*	**
		A. penicillioides	*	*
		A. poae	**	-
	Fusarium	*F. graminearum*	**	***
		F. verticillioides	*	*
	Penicillium	*Penicillium spp.*	**	*
		P. polonicum	-	*
		P. crustosum	-	*
	Other	*Rhizopus spp.*	+	-
		Unidentified microbe	+	-
		Yeast	+	+

KEY

*	100 – 300 x 10^4 CFU's
**	300 – 500 x 10^4 CFU's
***	>500 x 10^4 CFU's
+	Positive only

Using TLC, all samples in both categories tested positive for four mycotoxins (Table 3). The PB samples appeared to fare worse than GB samples particularly in terms of AF and ZEA concentrations. HPLC analysis investigated AF for AFB_1 and AFB_2 while FB was evaluated for FB_1 and FB_2 besides OTA and ZEA. Both PB and GB failed the limits set by the Fertilizer, Farm Feeds, Agricultural Remedies and Stock remedies Act No.36 of 1947 of 10ppb (1 ppb = 1µg/L) for total aflatoxins (South African Government 2009).

Table 3. Showing the results of TLC characterisation

		Premium	Grocery
TLC Characterisation	*Aflatoxin*	***	**
	Fumonisin	*	**
	Ochratoxin A	**	**
	Zearalenone	**	*

The levels of AFs (Table 4) detected in the PB was over the set limits for both AFB_1 (125.02ppb) and AFB_2 (11.77ppb) but GB only exceeded the limits for AFB_1 (41.57ppb). The amounts of both AFB_1 and AFB_2 were statistically significantly higher in PB as compared to GB (AFB_1, $p = 0.0087$; AFB_2, $p = 0.0091$; respectively). *Fusarium graminearum* was predominantly isolated in both categories; however, HPLC analysis indicated that the GB had exceedingly high concentrations of both FB1 (202.53ppb) and FB2 (118.37ppb), none failing the limit set by the Food and Drug Administration of 10,000ppb (FDA, 2001). The amounts of both FB_1 and FB_2 were significantly higher in GB as compared to PB (FB_1, $p = 0.028$; FB_2, $p = 0.0041$; respectively). OTA and ZEA levels were significantly higher in PB as compared to GB ($p = 0.0196$ for OTA and $p = 0.0060$ for ZEA) (Table 4).

Table 4. Showing the results of the HPLC quantitation of mycotoxins in cat feed extracts.

	AFB_1	AFB_2	FB_1	FB_2	OTA	ZEA
Premium	125,02**	11,77*	9,98	5,45	1,32	9,1*
Grocery	41,57	6,3	202,53***	118,37***	0,74	2,27

*Statistical significance

In summary, the PB fared worse than the GB in its AF, OTA and ZEA contamination, whilst GB contained much higher levels of FB than PB.

Discussion

Cats are obligatory carnivores and require taurine in their diets. A good animal protein source will provide the taurine required for a cat's good health. The presence of high amounts of mycotoxins in commercial cat diets is indicative of a high cereal content. PBs are perceived as better quality with superior nutrition than GBs, but they present a mycotoxin risk due to their high levels of cereal content. A study in Brazil showed a high correlation between diets rich in grains with a reduced immunity to infections in domestic animals (de Souza & Scussel 2012). Clinical signs described for dogs with aflatoxicosis are depression, anorexia, weakness, icterus and sudden death (Arnot *et al.* 2012; Ketterer *et al.* 1975; Stenske *et al.* 2006). Cats with sub-acute aflatoxicosis often show signs of lethargy, anorexia and progressive weight loss. Cats have lower susceptibility to mycotoxicosis than dogs but continuous exposure to low concentrations of mycotoxins in the feed can induce accumulative effects leading to chronic liver and kidney damage (Dereszynski et al. 2008).

The PB samples revealed a higher count of *A. flavus* CFUs than the GB samples. This finding is, however, not surprising as these are ubiquitous soil fungi and common contaminants of corn, groundnuts and other cereal grains used in animal feed production (Leung, Díaz-Llano & Smith 2006) and often implicated in aflatoxicosis. In addition, *Fusarium graminearum* was

detected at higher concentrations in the GB samples and are associated with FB, fusaric acid and ZEA production. *Penicillum* species were less commonly noted but PB samples showed higher concentrations than the GB samples. *Penicillium* spp. produce tremorgenic mycotoxins such as roguefortine and penitrem A. These mycotoxins induce tremorgenic mycotoxicosis particularly in canines characterised by acute abdominal pain, salivation, vomiting, fever, muscle tremors with hyperaesthesia, seizures and sometimes even death (Hocking, Holds & Tobin 1988; Naude *et al*. 2002; Young *et al*. 2003). OTA is produced by a number of *Aspergillus* and *Penicillum* species while ZEA is produced by *Fusarium* species (Leung *et al*. 2006; Shephard & Sewram 2004). OTA and ZEA were detected (by TLC and quantified by HPLC) at very low levels, but various studies have described toxicity at these reported levels (Leung et al. 2006).The role of *Fusarium* mycotoxins in animal health is particularly important economically as they have been implicated in infertility and reproductive dysfunction in sheep, cattle and pigs. Poultry are particularly affected with loss in weight, egg production and gastro-intestinal lesions (Antonissen *et al*. 2014; D'Mello et al., 1999; Placinta et al., 1999).

A mycotoxin mix of FB_1, FB_2, OTA, and ZEA together with AFs may present a higher risk to illness or mycotoxicosis. Many researchers have reported the simultaneous occurrence of several mycotoxins in feed and feed ingredients (Fox, Hodgkins & Smart 2012; Mwanza 2007; Tulpule 1981). This potent mycotoxin combination may result in synergistic action and potentiate effects that support the multi aetiological theory (Boermans & Leung 2007; Creppy *et al*. 2004; Mwanza *et al*. 2013; Ryu, Jackson & Bullerman 2002).

Irrespective of marketing channels, all products were contaminated with mycotoxins. The mean AF concentration across the various brands indicates that all products failed the prescribed limit (10ppb; by the Fertilizer, Farm Feeds, Agricultural Remedies and Stock remedies Act No.36 of 1947 {South African Government, 2009). The long term exposure of cats to mycotoxins may be implicated in numerous clinical conditions such as neoplasia, reduced immunity, poor growth and fertility (de Souza & Scussel 2012).

Conclusion

PBs are marketed as superior feeds but their cereal content makes them susceptible to mycotoxin contamination. Many PBs are imported and the higher mycotoxin content may be attributed to lengthy transport in containers on the high seas and high humidity. Though cats may appear to be less susceptible to mycotoxicosis, the risk of long-term exposure to mycotoxins coupled with poor health or concurrent disease could result in increased susceptibility. Further in vivo studies are required in evaluating feline susceptibility to mycotoxins.

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Chapter 5-Paper 3

Toxicity Assessment of Mycotoxins Extracted from Contaminated Commercial Dog Pelleted Feed on Canine Blood Mononuclear Cells

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Abstract

Raw ingredients of pet food are often contaminated with mycotoxins. This is a serious health problem to pets and causes emotional and economical stress to the pet owners. The aim of this study was to determine the immunotoxicity of the most common mycotoxins (aflatoxin, fumonisin, ochratoxin A and zearalenone) by examining 20 samples of extruded dry dog food found on the South African market [10 samples from standard grocery store lines (SB), 10 from premium veterinarian lines (PB)]. Pelleted dog food was subjected to extraction protocols optimized for the above mentioned mycotoxins. Dog lymphocytes were treated with the extracts (24 hour incubation and final concentration 40µg/ml) to determine cell viability, mitochondrial function, oxidative stress, and markers of cell death using spectrophotometry, luminometry and flow cytometry. Malondialdehyde, a marker of oxidative stress showed no significant difference between SB and PB, however, GSH was significantly depleted in SB extract treatments. Markers of apoptosis (phosphatidylserine externalization) and necrosis (propidium iodide incorporation) were elevated in both food lines when compared to untreated control cells, interestingly SB extracts were significantly higher than PB. We also observed decreased ATP levels and increased mitochondrial depolarization in cells treated with both lines of feed with SB showing the greatest differences when compared to the control. This study provides evidence that irrespective of price, quality or marketing channels, pet foods present a high risk of mycotoxin contamination. Though in this study PB fared better than SB in regards to cell toxicity, there is a multitude of other factors that need to be studied which may have an influence on other negative outcomes.

Introduction

Mycotoxins are secondary metabolites produced by molds and exert toxic effects on animals and humans (Peraica et al., 1999). They are ubiquitously produced by *Fusarium*, *Aspergillus* and *Penicillium* mold species which are common parasitizing agents of agricultural produce (Zain, 2011). Susceptibility to mycotoxicoses is dependent on animal species and on factors such as type of mycotoxin, concentration and duration of exposure. Due to the diverse molecular structures, mycotoxins possess a myriad of biological activities and are known to affect whole organ systems (Zain, 2011). Outbreaks of mycotoxicosis in humans have been regularly reported since early history. Egotism (commonly described as "Saint Anthony's Fire") occurred frequently in the middle ages, with major outbreaks accounting for a death rate of between 10 and 20 percent (Lee, 2009). The outbreak of "Turkey X disease" in Britain (Blount, 1961) was linked to a peanut meal contaminated with aflatoxins from *Aspergillus flavus*. This discovery highlighted the pathogenic potential of mycotoxicosis and soon the mycotoxin category was made to include a number of secondary metabolites such as orchratoxin (OTA) as well as previously isolated compounds such as patulin (PAT) (Bennett and Klich, 2003).

Mycotoxicosis in animal research has focused generally on farm and food animals (Ledoux et al., 1992, Lippold et al., 1992). Mycotoxicosis in these animals has economic implications as people often sustain losses to livestock and production of animal by-products (eggs, milk). Reports suggest that an indirect risk due to the carryover of toxins and their metabolites to edible animal products exists (Yiannikouris and Jouany, 2002, Zain, 2011, Völkel et al., 2011).

Several studies have implicated mycotoxins in cytotoxic and immunotoxic outcomes in animals (Pierron et al., 2016, Zain, 2011). A number of immunotoxic studies have focused on farm animals and laboratory animals (Rotimi et al., 2016, Mwanza et al., 2009) with pigs appearing to the most susceptible to mycotoxicosis (Mwanza et al., 2009). Of particular importance are the toxins aflatoxin (AF), fumonisin (FB), OTA and zearalenone (ZEA) as these are the most common contaminants of feed raw ingredients. Limited cytotoxicity studies have been performed in the realm of companion animals but many studies have recorded post mortem pathology and toxicology which is associated with mycotoxin poisoning (Greene et al., 1977, Hughes et al., 1999, Dereszynski et al., 2008, Bastianello et al., 1987).

The contamination of agricultural ingredients used in the pet food manufacturing process poses a serious health risk to companion animals. Cereals and cereal by-products contaminated with mycotoxins are commonly used in the manufacturing of pet foods and have been implicated in outbreaks of mycotoxicosis in several countries (Boermans and Leung, 2007, Arnot et al., 2012, Scudamore et al., 1997). Pet foods are manufactured as moist, semi-moist or dry extruded pelleted products and must meet certain nutritional standards set by consumer watch bodies such as the Association of American Feed Control Officials (AAFCO) or governmental organisations. However, none of these organisations have set prescribed mycotoxin limits for the final product, though limits are imposed for the core nutritional ingredients (Streit et al., 2012).

The increased humanization and attention to animal well-fare has resulted in greater emphasis being placed on the potential risk of mycotoxins in companion animals following numerous recalls of different pet food products in recent years. Most studies regarding companion animals and diets have been centred on feed analysis for toxin content (Bissoqui et al., 2016, Scudamore et al., 1997, Maia and Siqueira, 2002) or as reactionary investigations to outbreaks of mycotoxicoses (Arnot et al., 2012, Bastianello et al., 1987).

Aggressive marketing strategies used in the growing pet food industry, often coax the consumer to make impulse purchases based on packaging, labelling and price. Words such as "Ultra-Premium", "Gourmet" and "Superior" are normally coupled with higher prices and lures the consumer into perceiving that these foods are of a better quality (Fox et al., 2012). The dietary information on packaging often provides good information on ingredients and nutrient content but never on the bioavailability of these nutrients. Furthermore, no information on potential fungal containment or mycotoxin content is provided. A limited number of studies have looked at the comparison between premium diets (veterinary channel) and standard diets (supermarket or grocery channel). A recent study in Italy concluded that standard brands were more contaminated with mycotoxins than the premium brands (Gazzotti et al., 2015), following the stereotypical public perception. However, the differential effects of these diets on immunotoxicity has not been evaluated to our knowledge.

Consumption of mycotoxins can lead to impaired immunity resistance to pathogens as well as reactivation of chronic infections. The sensitivity of the immune system to mycotoxin induced

suppression arises from the vulnerability of the contiuous proliferation and differentiation of cells that contribute to immune-mediated activities and regulate signalling between cellular and humoral components (Oswald et al., 2005). Recently we showed using high performance liquid chromatography (HPLC) that mycotoxin contamination was prevalent in both PB and SB pet foods in Durban, South Africa (Singh and Chuturgoon, 2017).

Our study questions the validity of the perceived quality and safety of premium brand diets as akin to standard brand diets, by examining cytotoxic and immunotoxic effects of mycotoxins present in these feed samples on dog lymphocytes. This investigation also sits well with the emerging interest in One Health that gives due credence to plants, man, animal and the environment (Dhama et al., 2013). We investigated the effects of mycotoxin extracts derived from PB and SB dog food on isolated canine peripheral blood mononuclear cells (PBMCs).

Materials and Methods

Ethical approval and blood sample collection

This study was approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal, Durban, South Africa (Reference: 043/11/Animal). Written owner consent was obtained prior to blood collection from dogs. A health check was performed by taking history, temperature, pulse rate and heart rate. A blood smear was examined for parasites, blood pathology and routine perusal. Two German Shepard Dogs (30-35 kg body weight), fed on a PB diet, were bled via venipuncture of the cephalic vein on the right forelimb. The area was shaved and swabbed with 0.5% chlorhexidine gluconate in alcohol solution (Biotane – Dismed Pharmaceuticals), and 20 mL of whole blood was removed with a 21"G 25 mm needle and syringe. Blood was dispensed into heparinized tubes and was transported on ice to the laboratory for further analysis.

Materials

Cell culture reagents for peripheral blood mononuclear cell (PBMC) maintenance were purchased from Sigma Aldrich (Johannesburg, SA). The cell culture media was purchased from Scientific Group (Johannesburg, SA). Luminometry reagents were obtained from Promega (Madison, USA).

Mycotoxin extraction and clean-up of feed samples

A total of 20 samples of pelleted dog food were purchased per category (PB and SB) from various retail outlets (Durban, South Africa). These were subjected to sample preparation and extraction protocols [acid (A), neutral (N) and acid-neutral (AN) fraction of mycotoxins] that we have previously reported (Singh and Chuturgoon, 2017). The protocols were optimized for the extraction of AF, FB, OTA and ZEA. The mycotoxin containing extracts were standardized (40 µg/mL) using (HPLC) and used in treatment of isolated canine PBMCs.

Extraction of peripheral blood mononuclear cells

Buffy coats containing PBMCs were extracted from heparinized whole canine blood by differential centrifugation. Whole blood (5 mL) was isolated from two healthy male German Shepard dogs and was carefully layered onto equivolume Histopaque-1077 (Sigma-Aldrich, SA) in 15 mL polypropylene tube, centrifuged at 400 g for 30 minutes (min) at room temperature (RT). Following this, buffy coats were aspirated into new polypropylene tubes and washed twice in phosphate buffered saline (PBS) (by centrifugation at 400 g, for 20 min, RT). Cell density was determined by trypan blue exclusion and manually counted using a haemocytometer. Isolated PBMCs were maintained at 37 ^{0}C with 5% CO_2 in complete culture media (CCM, RPMI 1640 medium supplemented with 10% FCS, 1% L-glutamine and 1% penicillin-streptomycin). Cells were adjusted to a density of 1×10^6 cells/mL.

Treatment protocol

PBMCs were seeded into a 96 well microtitre plate (3.0×10^5 cells/well) and treated with 40µg/mL (final concentration) of the A, N and AN fractions of mycotoxin extracts. The concentration used was based on the study by Mulunda and Dutton (2014) who showed that mycotoxins at a dose of 40 µg/mL gave a better combinational response as compared to a lower dose in PBMCs (Mulunda and Dutton, 2014). The results were based on the MTT assay that indicated a 5 µg/mL concentration of mycotoxin displayed antagonistic effects. We therefore used the 40 µg/mL dose for our study.

Cells were incubated for 24 hours (hr) at 37°C, and this treatment protocol was followed for all subsequent assays.

Mitochondrial depolarization and function

Mitochondrial depolarization was assessed using the JC-1 Mitoscreen assay (BD Biosciences, Johannesburg, SA) by following the manufacturer's guidelines. Briefly, 5×10^5 PBMCs were treated with PB and SB mycotoxin extracts (A, N and AN) before being transferred to polystyrene flow cytometry tubes and incubated for 15 mins at RT with 150 μL of JC-1 dye. Thereafter, PBMCs were washed twice in JC-1 wash buffer (1×) and re-suspended in 200 μL flow cytometry sheath fluid. Labelled PBMCs were detected by fluorescence-activated cell sorting (FACS). Data was analyzed with FlowJo 7.1 software and expressed as a percentage of cells containing depolarized mitochondria.

Mitochondrial function was assessed by measuring intracellular ATP levels. PBMCs (1×10^5 cells/well) were seeded into a white luminometry plate and then treated with each of the A, N and AN mycotoxin extracts. ATP CellTitre Glo (Promega, Madison, USA) reagent (10 μL) was added and allowed to react in the dark (10 min, RT). Following incubation, the luminescent signal was detected with a microplate luminometer (Turner Biosystems, Sunnyvale, USA). Results were expressed as mean relative light units (RLU).

Evaluation of oxidative stress and anti-oxidant potential

Oxidative damage was assessed using the thiobarbituric acid assay which quantifies the levels of malondialdehyde (MDA), a by-product of lipid peroxidation. Supernatants from PBMCs treated with mycotoxin extracts of SB, PB and untreated controls were added to test tubes (200 μL), followed by the addition of 2% H_3PO_4 (200 μL), 7% H_3PO_4 (200 μL) and thiobarbituric acid/butylated hydroxytoluene solution (400 μL). A positive control of MDA was prepared. All samples were adjusted to pH 1.5 and boiled (15 min). Once cooled, butanol (1.5 mL) was added to each test tube, vortexed and allowed to separate into distinct phases. The upper phase (100 μL) from each sample was dispensed into a 96-well microtitre plate in five replicates. The optical density was measured on a spectrophotometer at 532 nm with a reference wavelength of 600 nm. The mean optical density of five samples per treatment was calculated and divided by the absorption coefficient (156 mM^{-1}). Results were expressed as MDA concentration (μM).

Anti-oxidant potential was assessed using the Glutathione-Glo™ Assay (Promega, Madison, USA). Cell suspensions from each extract (20,000 cells; 50 μL) was added in triplicate to the wells of an opaque 96-well microtitre plate. GSH standards (0-50 μM) were prepared from a

5 mM stock diluted in de-ionized water. Each GSH standard (50 µl) and sample had 50 µL of the GSH-Glo™ reagent added per well and incubated in the dark (30 min, RT). Reconstituted Luciferin Detection Reagent (50 µl) was added per well and incubated (15 min, RT). The luminescence was measured on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). The standards were used to draw a standard curve from which GSH concentrations in samples could be extrapolated. Results are expressed as µM.

Cell death parameters

The Annexin-V FITC apoptosis detection kit (Roche, South Africa) was used to determine phosphatidylserine (PS) externalization as a marker of apoptosis. Briefly, 1×10^5 PBMC's were treated with PB and SB mycotoxin extracts (A, N and AN). Cells were transferred to polystyrene flow cytometry tubes and stained with 5 µL of both the Annexin-V FITC and propidium iodide (PI, measure of necrosis) components (15 min in dark, RT). Thereafter, the samples were supplemented with 400 µL of Annexin-V Binding Buffer (1×). Labelled PBMCs were detected by flow cytometry (FACS Calibur, BD Biosciences, Johannesburg, South Africa). Data was collected for 50,000 events per sample and analyzed using FlowJo 7.1 software (Tree Star Inc., Ashland, USA).

Caspases -8, -9 and -3/7 activities were measured using Caspase-Glo® assays (Promega, Madison, USA) according to manufacturer's guidelines. Approximately 1×10^5 PBMCs was treated with A, N and AN fractions of mycotoxins before being aliquoted into a white luminometer plate (triplicate). Thereafter, 10 µL of the Caspase-Glo® -8,-9,-3/7 reagents were added and allowed to react. Plates were agitated at low speed for 30 min (dark, RT). Following incubation, the luminescent signal produced by mono-oxygenation of amino-luciferin was measured with a microplate luminometer. The results are expressed as relative light units (RLU).

Statistical analysis

Statistical analyses were performed using GraphPad Prism v5.0 software (GraphPad Software Inc., La Jolla, USA). The data was expressed as mean ± standard deviation (SD). Statistical significance between groups was assessed using the one way ANOVA and student *t*-test at a 95% confidence interval. Statistical significance was set at a value of $p < 0.05$.

Results

Assessment of mitochondrial function in PBMCs

Following treatment with PB and SB derived mycotoxin feed extracts, mitochondrial function was assessed by measuring intracellular ATP levels and alterations to mitochondrial membrane potential.

Cellular ATP levels were decreased significantly for all fractions of mycotoxins tested in PBMCs following exposure to PB (A = $6.256 \times 10^5 \pm 0.746 \times 10^5$ RLU vs. $13.24 \times 10^5 \pm 0.7764 \times 10^5$ RLU, $p = 0.0074$; N = $5.138 \times 10^5 \pm 0.7767 \times 10^5$ RLU vs. $13.24 \times 10^5 \pm 0.7764 \times 10^5$ RLU, $p = 0.0052$; AN = $7.565 \times 10^5 \pm 0.8668 \times 10^5$ RLU vs. $13.24 \times 10^5 \pm 0.7764 \times 10^5$ RLU, $p = 0.0082$) and SB extracts (A = $7.136 \times 10^5 \pm 1.088 \times 10^5$ RLU vs. $13.24 \times 10^5 \pm 0.7764 \times 10^5$ RLU, $p = 0.0060$; N = $8.71 \times 10^5 \pm 0.7322 \times 10^5$ RLU vs. $13.24 \times 10^5 \pm 0.7764 \times 10^5$ RLU, $p = 0.0240$; AN = $6.733 \times 10^5 \pm 0.5353 \times 10^5$ RLU vs. $13.24 \times 10^5 \pm 0.7764 \times 10^5$ RLU, $p = 0.0204$) when compared to control cells. In addition, ATP levels were significantly increased in the SB N extract compared to the PB N extract ($p = 0.0041$) (Figure 1A).

Mitochondrial membrane potential was then assessed. PB extracts (A = 24.20 ± 3.349% vs. 9.390 ± 0.5%, $p = 0.0018$; N = 25.60 ± 4.023% vs. 9.390 ± 0.5%, $p = 0.0031$; AN = 15.65 ± 2.306% vs. 9.390 ± 0.5%, $p = 0.0567$) and SB extracts (A= 10.78 ± 0.2205% vs. 9.390 ± 0.5%, $p = 0.05$; N = 14.85 ± 1.263% vs. 9.390 ± 0.5%, $p = 0.0039$; AN = 15.45 ± 2.475% vs. 9.390 ± 0.5%, $p = 0.05$) significantly increased mitochondrial depolarization as compared to the controls (Figure 1B). Also, a significant increase in mitochondrial depolarization for the PB N and PB A fractions were observed when compared to the SB N and SB A treatments (Figure 1B).

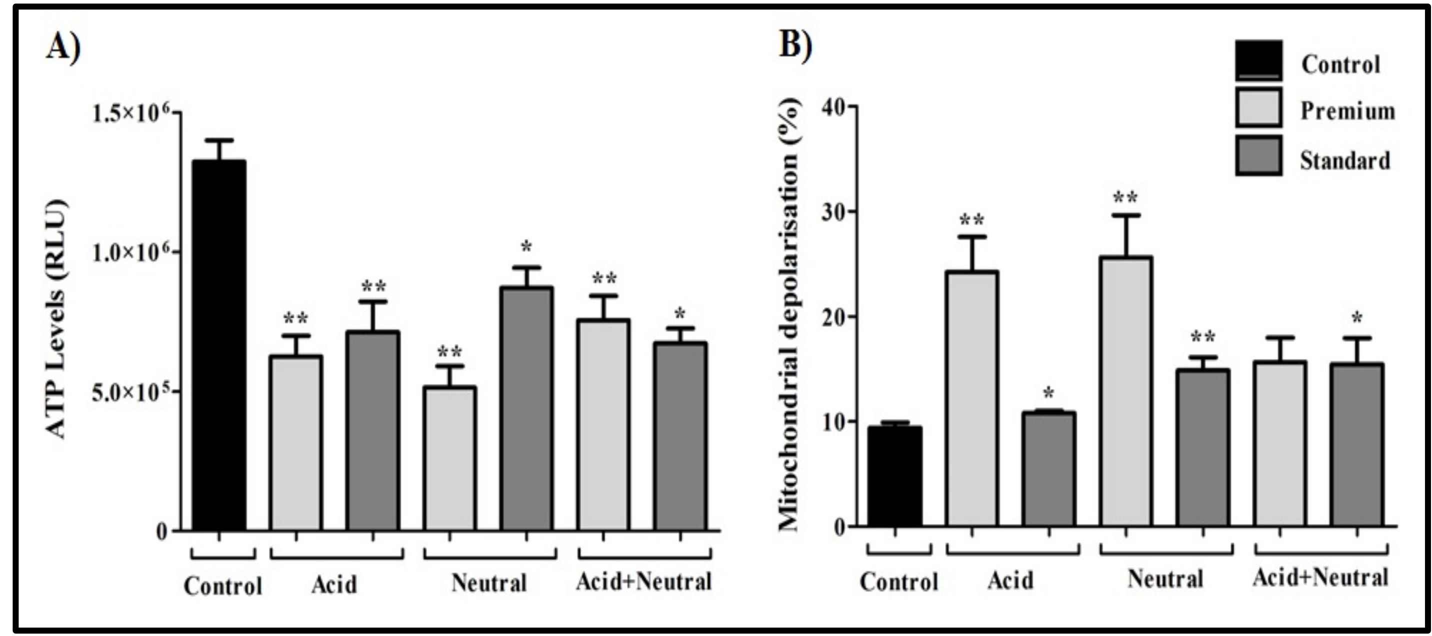

Figure 1: ATP levels (A) were significantly decreased in both categories of food brands (*p < 0.05). The percentage of depolarized mitochondria (B) was significantly higher for both categories of food compared to the controls, however, PB showed a greater increase in depolarization than SB in PBMCs following exposure to extracts (**p < 0.009). Data is presented as mean ± SD

Evaluation of oxidative stress and anti-oxidant potential

MDA (lipid peroxidation) and GSH (antioxidant potential) was measured in canine PBMCs. Levels of MDA after treatment with PB and SB feed extracts showed minimal differences (Figure 2A). However, GSH levels for both PB and SB extracts showed some significant trends. Only the PB N extract significantly depleted levels of GSH in PBMCs, whilst all SB extracts (A, N and AN) significantly decreased GSH levels as compared to controls (Figure 2B).

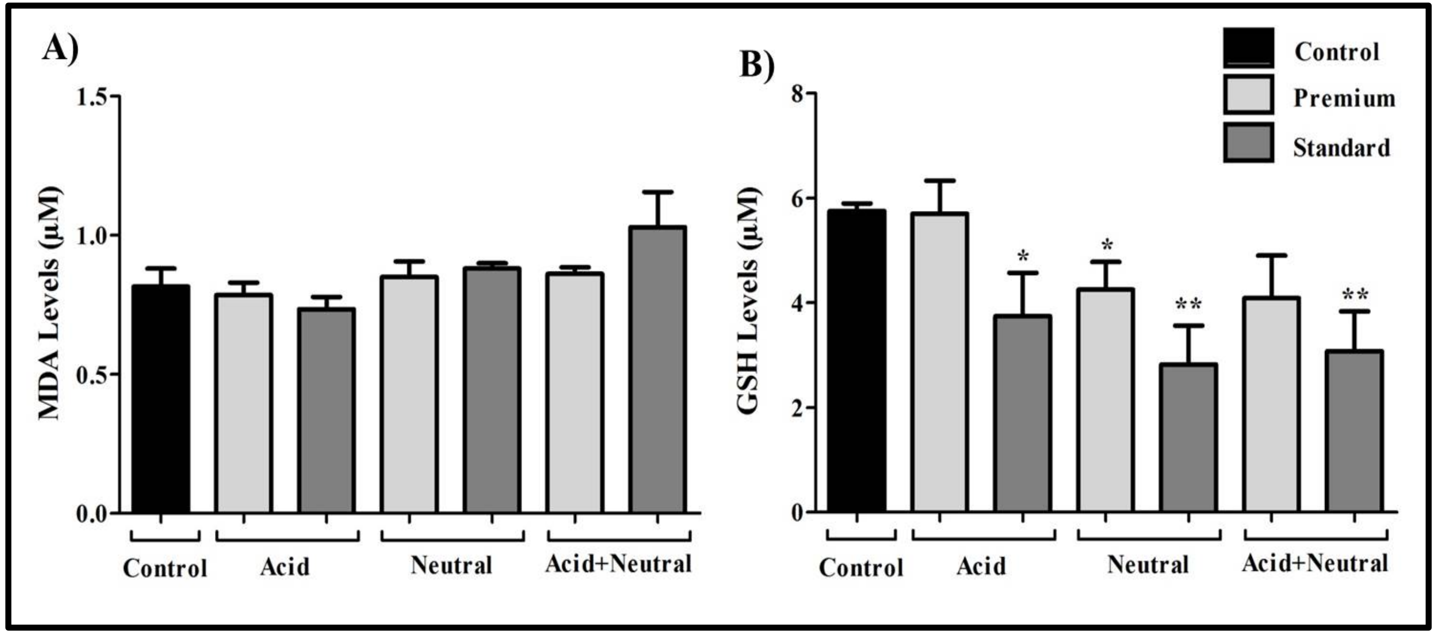

Figure 2: The levels of MDA (A) and GSH (**$p < 0.009$, B) in canine PBMCs after exposure to PB and SB feed extracts of mycotoxins (A, N, AN). Data is presented as mean ± SD.

Cell death parameters

Markers of cell death in canine PBMCs were assessed using flow cytometry. The levels of apoptosis in PBMCs were assessed after exposure to mycotoxin extracts of PB and SB feeds. All extracts (A, N, AN) of PB (2.06-fold, 1.32-fold and 1.63-fold, respectively) and SB (A, N, AN, 1.74-fold, 1.88-fold, and 2.35-fold, respectively) showed increased levels of apoptosis when compared to the untreated control, as indicated by higher percentages of PS externalization. In addition, the SB N extract was significantly higher when compared to the PB N extract ($p = 0.0060$) (Table 1). A similar trend was seen in the AN group, SB extracts showed a significantly increased levels of apoptosis as compared to PB extracts ($p = 0.0002$). Necrotic cell numbers were elevated for all extracts (PB: A – 2.28-fold; N – 3.63-fold; AN – 2.99-fold and SB: A – 2.14-fold; N– 2.42-fold; AN – 2.53-fold) compared to the control (Table 1).

Table 1: The effect of PB and SB feed extracts on apoptosis and necrosis in canine PBMCs

Type of death	Control	PB extracts (Mean ± SD)			SB extracts (Mean ± SD)		
		A	N	AN	A	N	AN
Apoptosis	7.900±0.645	16.28±0.9608	10.39±1.049	12.86±0.7278	13.76±1.986	14.81±0.915	18.60±0.8903
P value		0.0004***	0.0825	0.0070**	0.0229*	0.0008***	0.0002***
Necrosis	1.903±0.077	4.336±0.7459	6.902±1.399	5.695±0.7094	4.062±0.952	4.602±0.729	4.813±0.5615
P value		0.0101*	0.0060**	0.0005***	0.05*	0.0062**	0.0009***

*/**/***: significantly different compared to the control; PB: Premium brand; SB: Standard brand. Acid (A), neutral (N) and acid-neutral (AN) fraction of mycotoxin extracts. Data is presented as mean ± SD.

The initiator (-8 and -9) and executioner (-3/7) caspase activity was measured to assess whether the PB and SB extracts induced caspase-dependent apoptosis. Caspase-8 activity was decreased in the PB (A and N) and SB AN extracts when compared to the control (Table 2), however PB (AN) SB (A and N) extracts increased caspase-8 activity (Table 2). Caspase-9 activity was increased by all extracts (PB and SB, Table 2). Caspase-3/7 activity was significantly elevated by all extracts except SB A extract in comparison to the control (Table 2).

Table 2: PB and SB feed extract effect on caspase activity in PBMCs

Caspase ($x10^6$ RLU)	Control	PB extracts			SB extracts		
		Acid	Neutral	Acid+Neutral	Acid	Neutral	Acid+Neutral
-8	0.8474±00898	0.8088±0.0452	0.7473±0.0889	0.8899±0.03	0.8843±0.08	0.9881±0.02	0.7479±0.0276
***p*-value**		0.7671	0.4864	0.7341	0.7880	0.3739	0.4821
-9	0.1829±0.0204	0.2221±0.0075	0.2361±0.0157	0.2206±0.01	0.2256±0.00	0.2231±0.00	0.1963±0.0065
***p*-value**		0.3223	0.1749	0.3329	0.3095	0.3095	0.6439
-3/7	1.206±0.1167	1.332±0.0347	1.247±0.133	1.321±0.033	1.188±0.143	1.289±0.027	1.208±0.0222
***p*-value**		0.4896	0.8286	0.5174	0.9245	0.6164	0.9896

Discussion

Mycotoxins are ubiquitously present in feed and feed raw ingredients. Consumption of contaminated products by companion animals can result in mycotoxicoses and several outbreaks have already been reported (Arnot et al., 2012, Scudamore et al., 1997, Boermans

and Leung, 2007). Mycotoxins are known immune dysregulators in different animal species (Al-Anati and Petzinger, 2006, Hueza et al., 2014, Oswald et al., 2005). The immune system is highly susceptible to the toxic effects of mycotoxins, mainly due to continuous proliferation of cells that function in pathogen defence (Oswald et al., 2005). Dogs have become increasingly humanized and special attention has been directed to their nutrition and well-fare. While many studies have looked at the pathogenesis of mycotoxicoses in dogs as well as the content of mycotoxins in their food, no study to our knowledge has looked at the effects of PB and SB dog foods with regard to these mycotoxins on immunotoxicity. For our study, we used a combined mycotoxin concentration of 40 µg/mL (found in PB and SB extracts and determined from HPLC analysis) for the common mycotoxins such as AFB_1, AFB_2, FB_1, FB_2, OTA and ZEA. Further, a study that investigated the combined mycotoxin concentration of 40 µg/mL on PBMCs was shown to induce synergistic effects on symptomatology and severity as compared to effects observed during single intoxications by mycotoxins (Mulunda and Dutton, 2014). Dogs are exposed to varying levels of mycotoxins (AF, FB, OTA and ZEA) which contaminate their feed. Dogs exposed to commercial feed and bread contaminated with aflatoxin had blood levels ranging from 100 µg/g AFB_1 and 40 µg/g AFG_1 (Zain, 2011). Also symptoms of exposure to OTA in dogs occurred at 0.2-3 mg/kg. Bovine PBMCs exposed to AF (5, 20 µg/mL) and FB (35, 70 µg/mL) showed that it influenced ROS and antioxidant mRNA levels inducing cytotoxicity via oxidative stress (Bernabucci et al., 2011). OTA exposure (0.46 – 3000 ng/mL) to porcine mononuclear cells showed a reduction in immune function and cytotoxicity (Köhler et al., 2002). In addition, high concentrations of OTA (>20 µg/mL) inhibits human lymphocyte proliferation. Human leukocytes exposed to ZEA also showed a decrease in cell viability from 10-80 µg/mL (Viera-Limón et al., 2015). At 40 µg/mL, cell viability was below 50% indicating that ZEA is cytotoxic. In addition, at 40 µg/mL there was a significant increase in lipid peroxidation. Therefore, in our study, exposure of dog PBMCs to mycotoxin concentrations of 40 µg/mL is comparable to several studies and the apoptotic effects induced may guide proper management and quality control of commercial feed to mitigate exposure to these mycotoxins.

The use of canine PBMCs in our study is justified as they are composed of mainly lymphocytes (T cells such as CD4 and CD8 – 75%), B cells and natural killer cells (about 25% combined) and monocytes and macrophages. These cells (PBMCs) are critical in the immune system to fight infections and other foreign particles (Charoenpornsook et al., 1998).

Mitochondria function by generating ATP through oxidative phosphorylation in their inner membranes (Park et al., 2011). Intracellular ATP levels are useful in determining cellular toxicity, as ATP is essential for many cellular activities and its production is tightly regulated in healthy cells. Early cell damage often results in decreased ATP synthesis as well as rapid depletion of intracellular ATP (Malekinejad et al., 2015). All studied fractions regardless of brand channel significantly depleted intracellular ATP levels as compared to controls (Figure 1A). These results are supported by the high percentage of depolarized PBMC mitochondria induced by both SB and PB when compared to the controls (Figure 1B). Mitochondrial depolarization is triggered when the permeability transition pore of this organelle is opened resulting in decreased oxidative phosphorylation (Ly et al., 2003).

Mycotoxins are known to affect the production of ATP and cause mitochondrial dysfunction. Inhibition of ATP production and bioenergetic compromise are recognized as mechanisms of action for a variety of mycotoxins. Mitochondrial toxicity resulting from mycotoxins include membrane swelling, disruption of cristae, loss of transmembrane potential and direct inhibition of electron transport as well as formation of mitochondrial DNA adducts and inhibition of protein synthesis (Domijan and Abramov, 2011, Brewer et al., 2013, Doi and Uetsuka, 2011, Joo et al., 2013).

Mitochondrial dysfunction often leads to the generation of ROS. To protect macromolecules from the detrimental effects of ROS, cells have developed anti-oxidant defence systems that are capable of neutralizing excess ROS and prevent oxidative stress (Park et al., 2011). Oxidative stress occurs when the anti-oxidant capacity of cells is superseded by increased concentrations of ROS (Sies, 1991). The formation of MDA, a by-product of lipid peroxidation and marker of oxidative stress, is often elevated by mycotoxins (Viera-Limón et al., 2015, Klarić et al., 2013). Our results showed minor changes in MDA levels by SB and PB extracts (Figure 2A). The mild oxidative stress in comparison to previously published reports can be attributed to the cocktail of mycotoxins present in each fraction regardless of brand origin. The cocktail of mycotoxins may act synergistically to negate their combined effect on ROS production and subsequent lipid peroxidation. In contrast to pure and single mycotoxin feeding experiments, consumption of naturally contaminated multiple mycotoxins may provide more accurate information on the effects of mycotoxins on cell toxicity and oxidative stress. Indeed

mycotoxins have been shown to exhibit antagonistic effects; Tavares (2013) highlighted the potential of AF to decrease ROS levels in OTA exposed cells through competition for glutathione molecules leading to an antagonistic cytotoxic effect (Assunção et al., 2016). Other factors such as concentration of mycotoxin extracts in each fraction, type of cell and incubation period as well as competition for uptake of mycotoxins may contribute minimally to oxidative stress.

It is not clear whether mycotoxins stimulate lipid peroxidation directly by enhancing free radical production or if the increased lipid peroxidation is a result of downregulating anti-oxidant defence. We therefore measured the concentration of GSH, a potent intracellular anti-oxidant. Studies on rats showed an increase in MDA concentrations with a concomitant reduction in glutathione (GSH) levels (Martinez-Alfaro et al., 2006, Koksal et al., 2004). GSH is a potent anti-oxidant, but also participates is xenobiotic metabolism and cell signalling. GSH donates an electron to neutralize ROS and is oxidized to GSSG. GSH is re-generated from GSSG by glutathione reductase (GSR) using NADPH as an electron donor (Marí et al., 2009). All fractions decreased GSH concentrations in PBMCs; there were statistically significant changes in the N and AN fractions for PB. Similar changes for all fractions of SB were noted, however, greater reduction was seen in SB as compared to PB (Figure 2B).

Mycotoxins influence the intracellular levels of GSH by affecting transcription factors such as nuclear factor (erythroid-derived 2)-like 2 (Nrf2) which bind to antioxidant responsive element (ARE) that is needed for GSH biosynthesis. Cytokine deregulation also affects the synthesis of GSH (Guilford and Hope, 2014). The reduced levels of GSH in our study is supported by the depletion of ATP as GSH synthesis is dependent on two ATP requiring enzymatic reactions (Lu, 2009). These results are of particular concern as dogs have inherently lower levels of GSH and decreased activity of glutathione-S-transferase (Arnot et al., 2012).

Mitochondrial dysfunction and excessive ROS production are capable of inducing cell death. Phosphatidylserine externalization, an early marker of apoptotic cell death, was significantly increased by all fractions regardless of brand (Table 1). Apoptosis is induced by the activation of serine proteases called caspases. The mycotoxin PB A and N extracts reduced caspase 8 activity, but the same extracts increased caspase 8 activity by SB extracts. The response for the AN fraction was reversed for both PB and SB extracts. Though PB and SB showed increased apoptotic changes the contrary was seen for caspase 8 in some samples of both the PB and SB (Table 2). Caspase 8 activates caspase 3 as part of the extrinsic apoptotic pathway (Ly et al.,

2003); extrinsic apoptosis is associated with ligands binding to death receptors at the cell membrane. A possible reason for the differential activation of caspase 8 between brands and fractions may be attributed to the incorporation of mycotoxins into cell membranes, thereby causing damage. These changes are related to alterations of the fatty acid composition of membrane structures and peroxidation of long chain poly-unsaturated fatty acid residues inside membranes. This results in damage to membrane receptors, leading to aberrant second messenger systems; dysregulation of cellular pathways through inactivation of membrane-binding enzymes, and alterations in membrane permeability, flexibility and other characteristics needed for proper membrane function (Surai et al., 2008).

Similar trends were observed for caspase 9 activation with both PB and SB showing increased activity (Table 2). This is in keeping with the associated mitochondrial depolarization (Figure 1B). Opening of the mitochondrial permeability transition pore results in release of pro-apoptotic molecules such as SMAC/DIABLO and cytochrome C. These molecules are required for the activation of the intrinsic pathway of apoptosis (Ly et al., 2003). Caspase 3/7 activity (executioner caspases) was increased by all fractions of PB and SB, except SB A fraction (Table 2). The results indicate that both the extrinsic and intrinsic pathways of the apoptosis were initiated.

Investigations into cell death parameters revealed a marked increase in necrosis for all extracts in both PB and SB, with N extract of SB faring the worst (Table 1). Necrotic cell death results in damaged cell membranes and subsequent release of toxic substances that initiate an inflammatory response. Thus, cells undergoing necrosis cease production of proteins and ATP (Rosser and Gores, 1995). Contributing factors to necrosis may include ATP depletion, hypoxia and oxidative stress. In order to fully understand the implications of these findings it is essential to understand the energy state of the cell, especially in terms of ATP generation and mitochondrial function (Lieberthal et al., 1998).

Molecular changes induced by mycotoxins are seen at the cellular level before the first clinical symptoms may be noticed. Understanding the early cellular changes may assist in the early diagnosis and initiation of appropriate preventative and treatment measures to arrest pathogenesis. The potential danger of pet food being contaminated with high levels of mycotoxins poses a danger to companion animals via a variety of cytotoxic mechanisms.

Conclusion

This study proves that irrespective of price, quality or marketing channels, pet foods present a high risk of mycotoxin contamination. The high risk of mycotoxin contamination is associated with cellular and functional damage that influences physiological systems that result in ill health. Though in this study PB fared better than SB with regards to immunotoxicity, there is a multitude of other factors that need to be studied which may have an influence on other negative outcomes. This study provides important information to animal scientists and veterinarians that highlights the potential danger of mycotoxins and their adverse effects on the immune system. More importantly this study provides the consumer with the necessary information needed to inform their choices in their pet's nutrition.

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Chapter 6 - Paper 4

The immunotoxicity of commercial pelleted feline feed in peripheral blood mononuclear cells

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Abstract

Background: Mycotoxin contamination is a major concern in developing countries such as South Africa. Pelleted pet foods are often contaminated with mycotoxins that have a negative impact on pet health. This study investigated the immunotoxicity of some common mycotoxins found in commercial in cat food [6 samples from standard grocery store lines (SB) and 6 from premium veterinarian lines (PB)].

Methods: Samples of pelleted cat food were extracted for mycotoxins. Cat peripheral blood mononuclear cells (PBMCs) were isolated and treated with various feed extracts (24h) to determine oxidative stress (TBARS and GSH assay), mitochondrial integrity and cell death (Luminometry and Flow cytometry).

Results: Both PB and SB extracts significantly decreased ATP levels and increased mitochondrial depolarization except for the PB acid fraction. Lipid peroxidation was significantly increased in both PB and SB extracts with a concomitant decrease in GSH levels. Phosphatidylserine externalization and necrosis levels were increased in PB and SB extracts compared to the control. Executioner caspase-3/7 was also elevated following extract exposure except for the PB acid fraction.

Conclusion: PB and SB both display cell toxicity in cat lymphocytes however PB fared better with regards to immunotoxicity.

Background

There is an increasing awareness amongst pet owners with regards to pet nutrition, this coupled with numerous pet food recalls, has caused great public concern regarding the content of animal feeds. Many of these recalls were centred on aflatoxin outbreaks (Stenske et al., 2006, Newman et al., 2007, Arnot et al., 2012b). Most of the mycotoxin outbreaks appeared to have affected dogs and a few cases have been reported in other companion animals (Stenske et al., 2006, Newman et al., 2007, Arnot et al., 2012b). Many studies involving cat feed have illustrated significantly high levels of mycotoxins, which present a potential health risk (Barbosa et al., 2014, Crump, 2015). A recent study conducted by our group has demonstrated significant levels of mycotoxins in dog food sold via supermarket channels as well as in speciality brands which are sold via the veterinary channel (Singh and Chuturgoon, 2016)

Socio-economic change has been a great influencing factor in commodity prices. Consequently concerns over food security have caused prices to increase. In addition, the increase in pet ownership and increase demand for pet food has forced pet food companies to re-visit their feed formulations in order to keep prices under control in a competitive market. This has resulted in companies often substituting expensive animal protein with plant based protein and other by-products. The high percentage of plant based products in feed can result in fungal parasitization and increased risk of mycotoxin exposure (Boermans and Leung, 2007a).

Cats unlike dogs are obligate carnivores, requiring fresh protein to be incorporated into their diets to avoid numerous diseases. The amino acid taurine which is found in meat, is an essential dietary requirement to avoid long-term taurine deficiency that may result in retinal degeneration, loss of vision, and cardiac arrest (Knopf et al., 1978).

The requirements for superior proteins and supplementary vitamins in cat feeds can support accelerated growth of fungi. The potential for fungal growth and contamination maybe exacerbated by poor storage and handling of the product during import and export.

At the same time an adult cat's protein requirement exceeds a dog by 2-3 times (Zoran, 2002). This scenario of poor nutrition and formulation with the presence of a cocktail of mycotoxins (including masked mycotoxins) may create a synergism resulting in an increased

risk to disease (Stoev, 2013). This is of particular concern to the veterinarian, particularly in terms of the role that mycotoxins may play in the pathogenesis of disease.

The immunotoxic effects of mycotoxins have been studied in some animal species (Mwanza et al., 2009, Lioi et al., 2004, Stoev et al., 2012). Special attention has been placed on farm animals because of the potential health risks to humans. While minimal attention has been given to companion animals with more work being recorded in dogs than cats.(Little et al., 1991, Datz, 2010). Current regulations with regards to mycotoxin limits in pet food are non-existent, though regulatory bodies may prescribe levels in feed ingredients (FDA, 2001a, South African Government, 2009).This necessitates that cat owners are made aware of the potential dangers of the "unregulated" mycotoxin limits in feed that is consumed by their pets. In our study we intend to demonstrate the potential immunotoxic risk of mycotoxins in Premium brands (PB -veterinary marketing channel) with supermarket brands (SB -retail grocery channel) in isolated cat peripheral blood mononuclear cells (PBMC's).

Methods

This study was approved by the institutional Animal Research Ethics Committee of University of Kwa Zulu-Natal (Reference: 043/11/Animal). Feral cats presented at the Biomedical Resources Unit for sterilisation were used as donors. A health check was performed by taking history, temperature, pulse rate and heart rate. A blood smear was examined for parasites, blood pathology and routine perusal. Four cats of approximately 3-4 kilo-grams fed on sponsored diet PB were bled via venepuncture of right forelimb via the cephalic vein. The area was shaved and swabbed with 0.5% chlorhexidine gluconate in an alcohol solution (Biotane –Dismed Pharmaceuticals) and 5 mL of blood was removed with a 21"G 25 mm needle and syringe. Blood was collected in heparinised tubes. This was then transported, on ice, to the laboratory for further analysis.

Mycotoxin extraction and clean-up of feed samples

A total of 6 samples of pelleted cat food were purchased per category (PB and SB) from various retail outlets (Durban, South Africa). These were subjected to sample preparation and extraction protocols [acid (A), neutral (N) and acid-neutral (AN) fraction of mycotoxins] that we have previously reported (Singh and Chuturgoon, 2016). The protocols were optimized for the extraction of aflatoxins (AF), fumonisin B (FB), ochratoxin A (OTA) and zearalenone

(ZEA). Briefly, the AF, OTA and ZEA mycotoxin extraction were performed according to Patterson and Roberts, 1979. A mass of 25g of cat food was extracted with aqueous acetonitrile which contained potassium chloride (Singh and Chuturgoon, 2016). In addition, the mycotoxins were extracted with dichloromethane which contained sodium bicarbonate. This resulted in a neutral (N) fraction which was reacidified for an acid (A) fraction containing OTA. Further processing overnight with 30% aqueous acetone, the N fraction was then re-extracted in dichloromethane which contained AF and ZEA. The A and N fraction were then dried using nitrogen gas and subsequently stored in sealed vials. Extraction of FB was performed according to Shephard and Sewram (2004) with minor modifications. Briefly, 25g dog food was extracted in methanol and water in a ratio of 3:1 (50 ml) (Singh and Chuturgoon, 2016). It was allowed to mix on bench shaker for 1h and subsequently filtered (Whatman no. 2V filter paper). A volume of 5 mL methanol was used to pass the filtrate through a strong anion cartridge (SAX) column (Bond Elute, VARIAN, and SA). Subsequently, 5mL and then 8mL of methanol and water (3:1) were added. A final volume of 3mL methanol was then added. The absorbed FB was extracted in 10mL 1 % acetic acid in methanol, dried with nitrogen gas and subsequently stored in a sealed vial. To detect and quantify the mycotoxins TLC and HPLC were used respectively (Singh and Chuturgoon, 2016).

Extraction of peripheral blood mononuclear cells

Buffy coats containing peripheral blood mononuclear cells (PBMC's) were extracted from heparinised whole feline blood by differential centrifugation. Whole blood (5mL) was isolated from four healthy domestic short haired cats and carefully layered onto equi-volume Histopaque-1077 (Sigma-Aldrich, South Africa) in 15mL polypropylene tubes and centrifuged at 400 *g* for 30min at room temperature (RT). Following this, the buffy coats were aspirated into new polypropylene tubes and washed twice in phosphate buffered saline (PBS) (by centrifugation at 400 *g*, for 20min). Cell density was determined by trypan blue exclusion and manual cell counting on a haemocytometer adjusted to a density of 1×10^6 cells/mL.

Treatment protocol

PBMC's were seeded into a microtitre plate (3.0×10^5 cells/well) and treated in triplicate with serial dilutions of acid (A), neutral (N) and acid-neutral (A-N) fraction of mycotoxins

which was incubated for 24 hours at 37°C (final concentration 40µg/mL). This protocol was followed for all subsequent assays.

Intracellular ATP quantification

Quantities of 1×10^5 PBMC's were seeded into a white luminometry plate with (A, N and AN) extracts, to which the ATP CellTitre Glo (Promega, Madison, USA) reagent (10µL) was added and allowed to react in the dark for 10min at room temperature (RT). Following incubation, the luminescent signal proportional to the cellular ATP content was detected with a microplate luminometer (Turner Biosystems, Sunnyvale, USA). Results were expressed as mean relative light units (RLU).

Evaluation of oxidative stress and anti-oxidant potential

Oxidative damage was assessed using the thiobarbituric acid assay (TBARS) which quantifies the levels of malondialdehyde (MDA), a by-product of lipid peroxidation. Supernatants from PBMC's treated with mycotoxin extracts of SB, PB and untreated controls were added to test tubes (200µL), followed by the addition of 2% H_3PO_4 (200µL), 7% H_3PO_4 (200µL) and thiobarbituric acid/butylated hydroxytoluene solution (400µL). A positive control of MDA was prepared. All samples were adjusted to pH 1.5 and boiled (15min). Once cooled, butanol (1.5mL) was added to each test tube, vortexed and allowed to separate into distinct phases. The upper phase (100µL) from each sample was dispensed into a 96-well microtitre plate in five replicates. The optical density was measured on a spectrophotometer at 532nm with a reference wavelength of 600nm. The mean optical density of five samples per treatment was calculated and divided by the absorption coefficient (156 mM^{-1}). Results were expressed as MDA concentration (µM).

Anti-oxidant potential was assessed using the Glutathione-Glo™ Assay (Promega, Madison, USA). Cell suspensions from each extract (20,000 cells, 50µL) was added in triplicate to the wells of an opaque 96-well microtitre plate. GSH standards (0-50µM) were prepared from a 5 mM stock diluted in de-ionised water. Each GSH standard (50µL) and sample had 50µL of the GSH-Glo™ reagent added per well and incubated in the dark (30min, RT). Reconstituted Luciferin Detection Reagent (50µL) was added per well and incubated (15min, RT). The

luminescence was measured on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). The standards were used to draw a standard curve from which GSH concentrations in samples could be extrapolated. Results are expressed as µM.

Apoptosis studies

Detection of phosphatidylserine externalisation

An Annexin-V FITC apoptosis detection kit (Roche) was used to determine phosphatidylserine (PS) externalisation, 1×10^5 cells were treated with mycotoxin extracts (A, N and AN) treated and untreated cells were transferred to polystyrene cytometry tubes and stained with 5µL of both the Annexin-V FITC and propidium iodide (PI) components by incubation in the dark for 15min at RT. Thereafter, the samples were supplemented with 400µL of Annexin-V Binding Buffer (1×). Labelled PBMC's were detected by flow cytometry (FACS Calibur; BD Biosciences, Johannesburg, South Africa). Data was collected for 50,000 events per sample and analysed with FlowJo 7.1 software (Tree Star Inc., Ashland, USA).

Assessment of PBMC's mitochondrial membrane potential

The JC-1 Mitoscreen assay (BD Biosciences, Johannesburg, South Africa) was utilised to assess PBMC's mitochondrial membrane potential as per the manufacturer's guidelines. Briefly, 5×10^5 PBMC's were treated with mycotoxin extracts (A, N and AN) were transferred to polystyrene cytometry tubes and incubated (15min at RT) with 150µL of JC-1 dye. Thereafter, PBMC's were washed twice in JC-1 wash buffer (1×) and re-suspended in 200µL flow cytometry sheath fluid. Labelled PBMC's were enumerated by fluorescence-activated cell sorting (FACS). Data was analysed with FlowJo 7.1 software and expressed as a percentage of cells containing depolarised mitochondria.

Luminometric evaluation of caspase-3/7 activities

A quantity of 1×10^5 PBMC's were treated with extracts (A, N and AN) was aliquoted into a white luminometer plate, following which 10µL of the Caspase-Glo® 3/7 reagent (Promega) was added and allowed to react. Plates were agitated at low speed for 30min. Following

incubation, the luminescent signal produced by mono-oxygenation of amino-luciferin was measured with a microplate luminometer. The results were expressed as RLU.

Statistical analysis

GraphPad Prism v5.0 (GraphPad Software Inc., La Jolla, USA) was used for all statistical analysis. The data from the study was represented as a mean ± standard error of the mean (SEM). One way ANOVA and student *t*-test with 95% confidence interval was used for analysis and significance was set at $p < 0.05$.

Results

Mitochondrial integrity

The effect of the PB and SB extracts on mitochondrial integrity and function was assessed by quantifying intracellular adenosine triphosphate (ATP) levels and examining mitochondrial depolarisation (Figure 1).

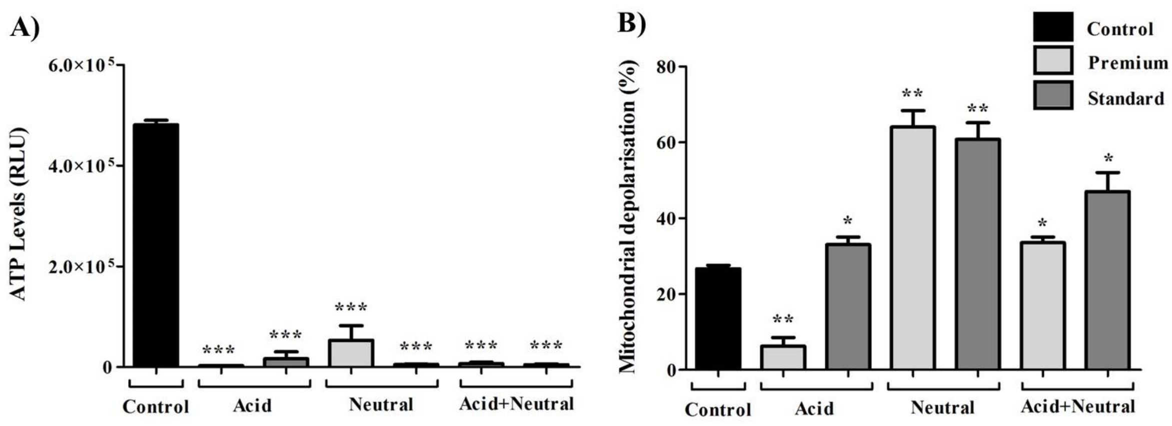

Figure 1: ATP levels (A) and mitochondrial depolarisation (B) in cat PBMC's after PB and SB extract exposure (*P < 0.05; **P < 0.01; ***P < 0.001: significantly different compared to the control; PB: Premium brand; SB: Standard)

ATP levels were significantly decreased in all extracts compared to the control. PB extracts showed a significant decrease in ATP levels: A (2893 ± 474.6RLU vs 480800 ± 9381RLU, *P*= 0.0004), N (53190 ± 28850RLU vs 480800 ± 9381RLU, *P* = 0.0008) and AN (6615 ±

3131RLU vs 480800 ± 9381RLU, $P = 0.0004$). Similarly, SB extracts (A: 16720 ± 13630RLU vs 480800 ± 9381RLU, $P < 0.0001$; N: 4979 ± 1394RLU vs 480800 ± 9381RLU, $P = 0.0004$; AN: 4826 ± 1534 vs 480800 ± 9381, $P = 0.0004$) had significantly reduced ATP levels.

Mitochondrial depolarisation was significantly decreased in the PB A extract compared to the control (6.2500 ± 2.2870% vs 26.6700 ± 0.8819%, $P = 0.0036$). A significant increase was seen in the PB N extract (64.0000 ± 4.3400% vs 26.6700 ± 0.8819, $P = 0.0035$). The PB AN extract also significantly increased mitochondrial depolarisation (33.50 ± 1.500% vs 26.6700 ± 0.8819, $P = 0.0234$). The SB A extract significantly increased mitochondrial depolarisation (33.00 ± 2.000% vs 26.6700 ± 0.8819%, $P = 0.0432$). Similarly, the SB N extract significantly increased the depolarisation of the mitochondria (60.7500 ± 4.4040% vs 26.6700 ± 0.8819%, $P = 0.0047$). The SB AN also significantly increased mitochondrial depolarisation (47.00 ± 5.000% vs 26.6700 ± 0.8819%, $P = 0.0137$).

Oxidative stress

The by-product of lipid peroxidation MDA, a marker of oxidative stress was assessed and shown in Figure 2A.

The extracts significantly increased MDA levels in cat PBMC's ($P = 0.0011$). A significant increase in MDA levels were seen in the A extracts of both PB (0.4050 ± 0.0608µM) and SB feeds (0.6067 ± 0.0851) (Figure 2A). Similarly, the N extracts increased MDA levels significantly when compared to the control (PB: 0.4050 ± 0.0698µM; SB: 0.2250 ± 0.0173µM) (Figure 2A). In addition, the AN extract also increased MDA levels in both PB (0.2125 ± 0.0328µM vs control: 0.0933 ± 0.0088µM, $P = 0.0391$) and SB (0.2900 ± 0.0285µM) feeds (Figure 2A).

The antioxidant, GSH was measured and showed that there was a significant decrease in GSH levels in both the PB (0.6150 ± 0.1887µM) and SB (0.8025 ± 0.3394µM vs) A extracts compared to the control (Figure 2B). Also, a significant decrease in GSH levels was seen in the PB N (1.1030 ± 0.1728µM) and PB AN (1.5580 ± 0.1307µM) extracts (Figure 2B). Similarly, a significant decrease in GSH levels for the SB N (0.6400 ± 0.2597µM) and SB AN (0.2625 ± 0.0894µM) extracts (Figure 2B).

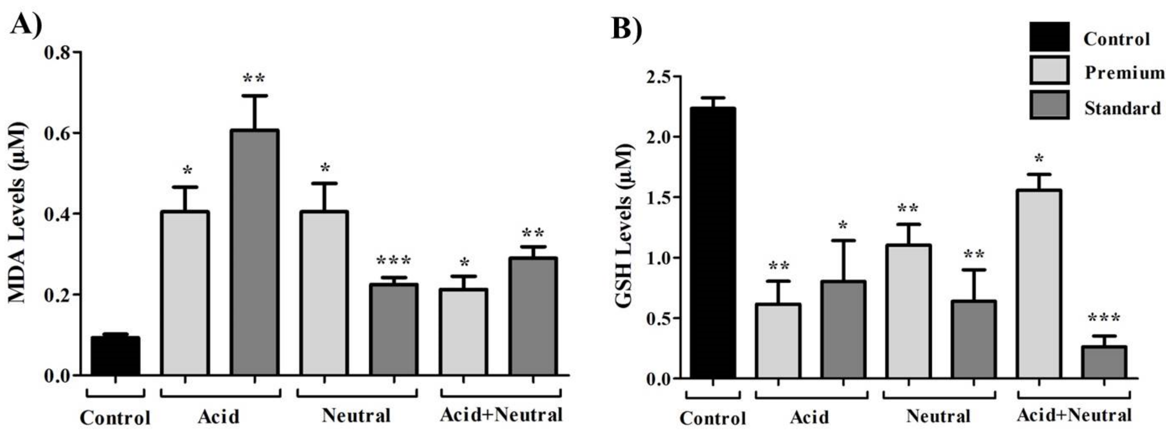

Figure 2: Lipid peroxidation – MDA levels (A) and GSH levels (B) after exposure to PB and SB feeds (*P < 0.05; **P < 0.01; ***P < 0.001: significantly different compared to the control; PB: Premium brand; SB: Standard)

Cell death markers

Apoptosis (phosphatidylserine externalisation) and necrosis levels were assessed in cat PBMC's and shown in table 1.

Table 1: PB and SB feed extract effect on apoptosis (phosphatidylserine externalisation) and necrosis levels in cat PBMC's

	Control	PB extracts (Mean ± SEM)			SB extracts (Mean ± SEM)		
		Acid	Neutral	Acid+Neutral	Acid	Neutral	Acid+Neutral
Apoptosis (%)	13.90 ± 0.2082	40.00 ± 11.06	14.33 ± 4.333	36.33 ± 6.489	28.67 ± 12.44	16.33 ± 0.8819	50.00 ± 5.686
***p*-value**		0.1423	0.9295	0.0745	0.3572	0.1152	0.0240*
Necrosis (%)	1.997 ± 0.0606	38.00 ± 17.09	31.25 ± 2.213	36.67 ± 3.383	80.67 ± 2.186	25.00 ± 2.887	38.25 ± 2.926

***p*-value**	0.1697	0.0009***	0.0094**	0.0008***	0.0154*	0.0011**

(*P< 0.05; **P< 0.01; ***P < 0.001: significantly different compared to the control; PB: Premium brand; SB: Standard brand)

PS externalisation, an early marker of apoptosis was assessed and showed that PB extracts (A: 2.88-fold; N: 1.03-fold; AN: 2.61-fold) increased apoptosis in cat PBMC's (Table 1). Similarly, the SB extracts (A: 2.06-fold; N: 1.18-fold; AN: 3.60-fold) also increased apoptosis (Table 1). In addition, necrosis levels were assessed and showed that the PB extracts (A: 19.03-fold; N: 15.65-fold; AN: 18.36-fold) significantly increased necrosis in cat PBMC's (Table 1). Also, the SB extracts showed a similar significant increase (A: 40.40-fold; N: 12.52-fold; AN: 19.15-fold) (Table 1).

Caspase activity in cat PBMC's

Execution of apoptosis occurs via caspase-3/7 and their activity was determined in cat PBMC's (Figure 3).

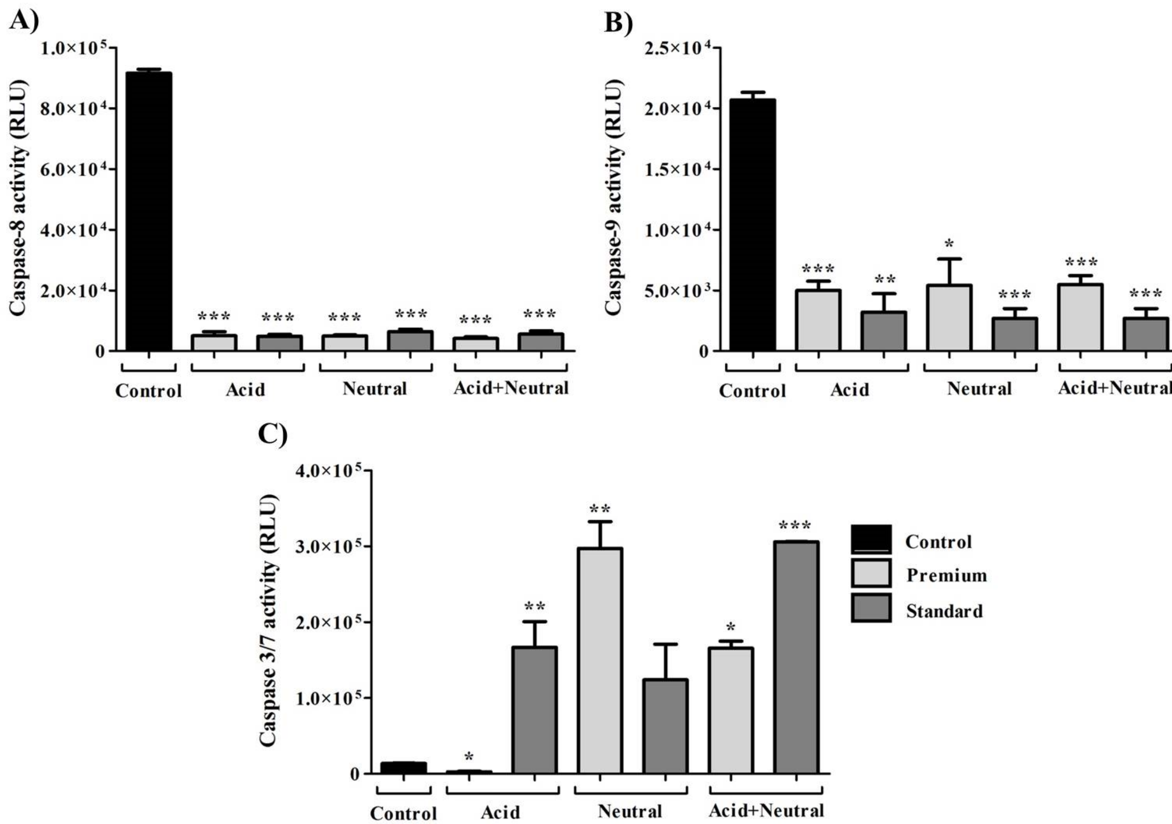

Figure 3: Caspase-3/7 activity after exposure to PB and SB extracts (*P < 0.05; **P < 0.01; ***P < 0.001: significantly different compared to the control; PB: Premium brand; SB: Standard).

Caspase-3/7 activity was significantly decreased in PB A extracts (2672 ± 737.5RLU vs 13720 ± 278.3RLU, $P = 0.0454$) and significantly increased in PB N (297200 ± 35330RLU vs 13720 ± 278.3RLU, $P = 0.0017$) and PB AN (165500 ± 9450RLU vs 13720 ± 278.3RLU, $P = 0.0396$) extracts. SB extracts (A: 166800 ± 34000RLU vs 13720 ± 278.3RLU, $P = 0.0091$; N: 124000 ± 46900RLU vs 13720 ± 278.3RLU, $P = 0.0511$; AN: 305900 ± 142.0RLU vs 13720 ± 278.3RLU, $P < 0.0001$) increased caspase-3/7 activity compared to the control (Figure 3).

Discussion

There are over 300 mycotoxins ubiquitously found in feed and feed raw ingredients due to fungal contamination. Feed contaminated with fungi can result in mycotoxicosis of which several outbreaks have been reported (Arnot et al., 2012a, Scudamore et al., 1997b, Boermans and Leung, 2007b). The nature and extent of the effects of mycotoxins on mammalian tissue depends on the concentration and length of exposure (Fink-Gremmels et al., 1995). Mycotoxins

display immunotoxic effects in a variety of mammalian species (Al-Anati and Petzinger, 2006, Hueza et al., 2014, Oswald et al., 2005). The immune system is highly susceptible to the toxic effects of mycotoxins either via acute (short period) or via chronic (longer periods) exposure. In general the effects of mycotoxins are mediated via damage to DNA, RNA and protein synthesis causing dysfunction in growth and development (Surai and Dvorska, 2005). Cats and dogs have become increasingly humanized in the modern era. This humanization has garnered extra attention to pet nutrition and welfare. Limited information is available with regards to commercial cat food content of mycotoxins (Singh 2016; Crump 2010). A study such as this fills an important gap in the understanding of the immunotoxicity of cat foods. Furthermore, a comparison of the supermarket or grocery channels (SB) as akin to the premium or veterinary channels (PB) would provide an additional dimension with regards to consumer awareness in pet food choices.

ATP is produced within the inner membrane of mitochondria through oxidative phosphorylation (Park et al., 2011) which is integral to cellular activities. This process is well regulated in healthy cells therefore intracellular ATP is useful in determining cellular toxicity. Early cellular damage often results in a cascade of reactions of which a reduction of ATP synthesis and rapid intracellular depletion of ATP occurs (Malekinejad et al., 2015). All studied fractions regardless of brand channel showed significantly depleted intracellular ATP levels as compared to control groups (Figure 1A). These results are supported by higher percentages of depolarized mitochondria in both SB and PB when compared to the control (Figure 1B), except in the A fraction of PB.

Mitochondrial depolarization is triggered when the permeability transition pore of this organelle is opened resulting in loss of oxidative phosphorylation (Ly et al., 2003). The production of ATP is altered by mycotoxins, which cause mitochondrial dysfunction. The resulting alteration inhibits ATP production and compromises cellular bioenergetics. This process is a mechanism of action for a myriad of mycotoxins including those described in this study. Mitochondrial abnormalities will result in some pathognomonic changes resulting from mycotoxins that will include membrane swelling, loss of transmembrane potential, disruption of cristae and direct inhibition of electron transportation, additionally as the formation of mitochondrial DNA adducts and an inhibition of protein synthesis (Domijan and Abramov, 2011, Brewer et al., 2013, Doi and Uetsuka, 2011, Joo et al., 2013).

Reactive oxygen species (ROS) arise as a result of mitochondrial dysfunction. As a protective mechanism, anti-oxidant defence systems are initiated by cells and is capable of neutralizing excess ROS, preventing oxidative stress (Park et al., 2011) thus protecting cellular macromolecules. When the anti-oxidant potential of cells is compromised by an increased concentration of ROS (Sies, 1991) oxidative stress occurs. The formation of MDA, a by-product of lipid peroxidation and marker of oxidative stress is often elevated after treatment with mycotoxins (Viera-Limón et al., 2015, Klarić et al., 2013). Our results show significant elevation in MDA levels for all fractions of SB and PB against the control, while SB fractions fared worse than PB except for the N fraction. (Figure 2A) suggesting SB has a higher level of mycotoxin contamination.

The oxidative stress in Figure 2A is in agreement with previous studies. In contrast to single mycotoxin feeding experiments, consumption of feed naturally contaminated with a cocktail of mycotoxins could provide better insights into the effects of mycotoxins on tissue toxicity and oxidative stress. Some mycotoxins are known to exhibit antagonistic effects wherein the potential of AF to deplete ROS levels in OTA exposed cells via competition for glutathione molecules resulting in an antagonistic effect (Assunção et al., 2016). A myriad of factors such as concentration of mycotoxin extracts in each fraction, incubation period, type of cells as well as competition for uptake of mycotoxins, may suggest a reduction in oxidative stress

It is not clear, whether mycotoxins stimulate lipid peroxidation directly by enhancing free radical production or if the increased lipid peroxidation is because of down regulation of the anti-oxidant defence. It seems likely that both processes are involved. Consequently, investigation of GSH concentrations is warranted. Studies in rats have eluded to an increase in MDA with a concomitant reduction in GSH (Martinez-Alfaro et al., 2006, Koksal et al., 2004). GSH is a potent anti-oxidant that participates is xenobiotic metabolism and cell signalling. The economic implications of lipid peroxidation effects were illustrated with studies in the agriculture-food industry and its detrimental effects in egg production and carcass quality (Dvorska et al., 2001, Castellini et al., 2002). This anti-oxidant molecule by donating an electron to neutralise ROS becoming oxidised (GSSG). GSH may be re-generated from GSSG with the enzyme glutathione reductase using NADPH as an electron donor (Marí et al., 2009). Concentration of GSH was decreased significantly in all fractions (A, N and A-N) of mycotoxins against the control. SB fraction fared worse than PB fraction for exception of A fraction (Figure 2B). However in a wider view, the results suggests that these mycotoxin

fractions regardless of brand or marketing channel elevates oxidative stress through dysfunctional mitochondria and depletion of anti-oxidant (GSH).

Mycotoxins have profound effects on the intracellular levels of GSH this maybe mediated via additive, synergistic or potentiated interactions (Eaton and Klaassen, 1996). Mycotoxins are able to affect transcription factors such as nuclear factor (erythroid-derived 2)-like 2 which bind to anti-oxidant responsive element which is needed for GSH biosynthesis. Cytokine deregulation also affects the synthesis of GSH (Guilford and Hope, 2014). The inhibition of GSH biosynthesis in our study is supported by the depletion of ATP, since GSH synthesis is dependent on two ATP requiring enzymatic reactions (Lu, 2009). In cats, the addition of taurine as a feline feed requirement may have a role in reducing the marked effects of mycotoxins (Timbrell et al., 1995) though it may also serve as nutrient substrate for accelerated fungal growth in cat food.

Mitochondrial dysfunction and excessive ROS production are capable of inducing cell death. Phosphatidylserine externalisation – an early marker of apoptotic cell death was significantly increased in the AN fraction of SB (Table 1).

Apoptosis is characterized by the activation of serine proteases called caspases. Caspases are subcategorized as initiator caspases, which include caspase -8 and caspase -9, and executioner caspases that include caspase -3 and caspase -7. Investigation of caspase -3/7 activity (executioner caspases) revealed increases in all fractions of PB and SB except PB acid fractions (Figure 3) confirming cat PBMC's cell death.

Investigations into cell death parameters further revealed a marked increase in necrosis for all extracts in both PB and SB fractions (Table 1). Necrotic cell death is synonymous with the release of toxic substances and can result in early initiation of an inflammatory response. When necrosis occurs the production of proteins and ATP ceases (Rosser and Gores, 1995). Contributing factors to necrosis may include ATP depletion, hypoxia and oxidative stress. In order to fully understand the implications of these findings it is essential to understand the energy state of the cell, especially in terms of ATP generation and mitochondrial function (Lieberthal et al., 1998).

Conclusion

The potential immunotoxicity of pet foods, both PB and SB, contaminated with high levels of mycotoxins poses a danger to felines. This study shows that irrespective of price, quality or marketing channels, pet foods are highly contaminated with mycotoxins with PB faring better than SB with regards to PBMC toxicity in cats.

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Chapter 7-Conclusion

The world human population is expected to reach 9.6 billion by 2050, consequently leading to the need for more food. This need will be significantly larger than present day production meets. It will require an increase in both plant and livestock production to meet these demands. With the increase of the human population and a concurrent anticipated increase in companion animal population, competition for both plant and animal protein for human and animal life is inevitable. The prudent and complete use of food stores and food sources will be required to create sustainability (Meeker and Meisinger, 2015).

The role animals' play in the lives' of humans (consumer) is complex which may include that of a friend, a family member or even an extension of the self (Hirschman, 1994). Hirschman seminal report in 1994 gave increasing attention to companion animals and their role in society. The increased attention to the human animal bond resulted in marketers appreciating the larger role that companion animal could play in directing consumer behaviour and influencing the bottom line (Aylesworth et al., 1999). These few events changed the face of the global pet food industry that is expected to reach global sales of US$74.8 billion, which is largely driven by increasing humanisation of pets with urbanisation, lifestyle changes and greater disposable incomes (Industry, 2016) These changes prompted the production of customised and premium diets to meet consumer nuances. The major players in the industry are Proctor and Gamble, Nestle, Mars and Colgate – Palmolive, amongst others immediately cottoned to the idea of premium brands and often selling these brands via the veterinary or pet speciality channel with the perception of superior product with better nutrition.

Though pet nutrition may have been reformulated and repackaged in many innovative ways the main ingredients continue to be the same, vegetable matter that supplemented with fats, meat meals and vitamins, all of which is highly susceptible to fungal contamination. Formulation of animal feed and diets is underpinned by the least cost formulation and often contains food materials that are rejected in human food production chain (Leung et al., 2006, Boermans and Leung, 2007a). Many studies have shown that fungal contamination (mycotoxins) is ubiquitous in processed (extruded and pelleted) feed (Sharma and Márquez, 2001, Mwanza et al., 2013, Buchanan et al., 2011). Mycotoxin contamination resulted in many global outbreaks of mycotoxicosis in companion animals with high mortality (Newman et al., 2007, Dereszynski et al., 2008b, Arnot et al., 2012b). In all cases, the impact on animal health was significant with symptoms of anorexia, vomiting, jaundice and even death. These changes

are mediated at the cellular level hence the role of mycotoxins as immune disruptors are widely recognised but studies are limited. Our study addressed both the cause and effect of mycotoxicosis, resulting in the following conclusion in both canine and felines studies.

Chapters 3 and 4 addressed the analysis of dog and cats foods, respectively, comparing SB (cheaper) with PB diets (expensive). The risk of mycotoxicosis in dog food is evident, though the general trend was that the SB scored higher as regards to mycotoxins content than the "speciality" PB. In the case of feline diets, the PB scored higher in AFB_1, which was not anticipated, based on the claims by PB companies, claiming better nutrition that caters for improved animal health. Furthermore, feline diets are expected to have lower contents of vegetable matter and more animal-based protein to satisfy the needs of the cat, being an obligate carnivore. The alternate theory been the higher content of AFB_1 in the PB diets maybe attributed to the superior nutrition available in the formulation that may provide excellent substrates for fungi to proliferate. This assumption may be coupled with the long journey that imported diets spend in the ship's hold, under high humidity and temperature, which supports fungal growth.

Chapters 5 and 6 investigated the toxicity of the feed extracts from commercially pelleted dog and cat food on peripheral blood mononuclear cells of the respective species. We found that dog PB extracts were less toxic then the dog SB on PBMC's. These results followed the trend of the analytical study wherein the presence of a mycotoxin mixture was lower in the PB samples when compared to SB samples. Our study clearly showed the profound action of the mycotoxin mix as immune disruptors via a myriad of biochemical tests. In the case of cat feed extracts we found that they have profound effects with both PB and SB as immune disruptors. In our cat food analysis, an unexpected occurrence of PB brands faring worse than SB for some mycotoxins paralleled such trends with its cytotoxic and immune toxic effects.

In our study, PB for both species were generally lower in mycotoxin content than SB but did not guarantee that the sample necessarily met the limit recommendations in ppb set by FDA or similar governmental and non-governmental organisation. Mycotoxin extracts for both

commercially pelleted brands (SB and PB) present an immunological, cytotoxic and disease risk to animals that consume it.

Future recommendations

Further research with wet food preparations together with studying individual mycotoxins and mycotoxin mixes is required. These findings regarding the effect of individual mycotoxins and mycotoxin mixes *in vitro* and *in vivo* studies would provide further information helping us to better understand the role of mycotoxins as immune disruptors.

The preparation of companion animal foods should be addressed with more scrutiny by looking at the harvesting, storage and use of good quality grain. Furthermore, a vigilant quality control systems and potential use of transgenic toxin free products (Thakare et al., 2017) could reduce mycotoxin risks. The implementation of the fundamentals of HACCP as quality control system using controlled inputs (ingredients) that influence the end (Horchner and Pointon, 2011) may provide a cost effective solution for the future.

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Appendix 1- Animal Ethics Approval

Research office
Animal Ethics Research Committee
Govan Mbeki Centre, Westville Campus,
University Road, Chiltern Hills, Westville, 3629, South Africa
Telephone 27 (031) 260-2273/35 Fax (031) 260-2384
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03 December 2010

Reference: 043/11/Animal

Dr S Singh
Biomedical Resource Unit
Medical Sciences
University of KwaZulu-Natal
WESTVILLE CAMPUS

Dear Dr Singh

Ethical Approval of Research Project on Animals

I have pleasure in informing you that on recommendation of the review panel, the Animal Ethics Sub-committee of the University Research and Ethics Committee has granted ethical approval for 2011 on the following project:

"A toxicological evaluation of commercial pet foods"

Yours sincerely

Prof. Theresa HT Coetzer (Chair)
ANIMAL RESEARCH ETHICS COMMITTEE

Cc Registrar
Research Office
Head of School (School office copy) Prof. A Chutturgoon

Founding Campuses:
- Edgewood
- Howard College
- Medical School
- Pietermaritzburg
- Westville

Appendix 2 – Fungal Contamination

Samples ID	Fungal contamination (cfuX10^4)
C1	Aspergillus parasiticus (310^4) A. flavus (510^4) Fusarium verticilioides (2. 10^4) yeast
C2	A. fumigatus (310^4) A. flavus (610^4) A. parasiticus (210^4.) A. niger (210^4) F. graminerium (210^4) Penicillium spp(410^4)
C3	A. niger (310^4) A. flavus (510^4) A. penicilioides (210^4) Peniicllium spp (310^4)
C4	A. fumigatus (410^4) A. flavus (610^4) A. parasiticus (310^4) A. niger (210^4) A. penicilioides (210^4) F. graminerium (310^4)
C5	A. fumigatus (410^4) A. flavus (610^4) A. parasiticus (310^4) A. niger (210^4) F. graminerium (310^4) Penicillium polonicum (2)
C6	A. fumigatus (310^4) A. flavus (410^4) A. parasiticus (310^4) A. niger (310^4) A. ochraceus (510^4) F. graminerium (810^4) F. verticilloides (310^4) Yeast
C7	A. fumigatus (210^4) A. flavus (410^4) A. parasiticus (3) A. niger (310^4) F. graminerium (110^4) F. verticilloides (210^4) Yeast unidentified Aspergillus (210^4)
C8	A. fumigatus (410^4) A. flavus (610^4) A. parasiticus (310^4) A. niger (210^4) A. penicilioides (210^4) Penicillium Polonicum (210^4) Unidentified penicellium spp(210^4) Yeast
C9	A. fumigatus (310^4) A. flavus (210^4) A. parasiticus (310^4) F. graminerium (310^4) F. verticilloides (210^4) Penicillium crostosum (310^4)
C10	A. parasiticus (310^4)

	A. flavus (210^{4}) F. graminerium (310^{4}) yeast
C11	A. fumigatus (410^{4}) A. flavus (610^{4}) A. parasiticus (310^{4}) A. niger (210^{4}) A. poae (310^{4}) F. graminerium (310^{4}) Penicillium spp(410^{4}) Yeast
C12	A. flavus (210^{4}) A. parasiticus (310^{4}) F. graminerium (310^{4}) Rhizopus spp (510^{4}) F. verticilloides (210^{4}) Penicillium spp(0.110^{4}) Yeast
D1	A. fumigatus (410^{4}) A. flavus (610^{4}) A. parasiticus (310^{4}) A. niger (210^{4}) F. graminerium (310^{4}) F. verticilloides (410^{4}) Penicillium spp2) Yeast
D2	A. fumigatus (210^{4}) A. flavus (710^{4}) A. parasiticus (3) A.ochraceus (210^{4}) F. graminerium (310^{4}) Penicillium spp(410^{4}) Yeast
D3	A. fumigatus (410^{4}) A. flavus (610^{4}) A. parasiticus (310^{4}) A. niger (210^{4}) F. graminerium (310^{4}) Penicillium spp (310^{4}) Yeast
D4	A. fumigatus (710^{4}) A. parasiticus (310^{4}) A. penicillioides (310^{4}) F. graminerium (210^{4}) Yeast
D5	A. flavus (410^{4}) A. parasiticus (310^{4}) Rhizopus spp F. graminerium (310^{4}) Yeast
D6	A. flavus (1410^{4}) A. parasiticus 410^{4})
D7	F. graminerium (210^{4}) Yeast infection
D8	A. flavus (210^{4}) A. parasiticus (210^{4}) A. fumigatus (510^{4}) A. niger (210^{4})

	F. verticilloides (210^{3}) F. graminerium (310^{4}) Penicillium spp(410^{4}) Yeast
D9	A. fumigatus (410^{4}) A. flavus (710^{4}) A. parasiticus (310^{4}) A. niger (210^{4}) F. graminerium (310^{4}) Penicillium spp(410^{4}) Yeast
D10	A. fumigatus (210^{4}) A. flavus (410^{4}) A. parasiticus (1) A. niveus (210^{4}) F. graminerium (310^{4}) Yeast
D11	A. parasiticus (310^{4}) A. niger (210^{4}) F. graminerium (310^{4}) Penicillium spp (410^{4}) Yeast
D12	A. fumigatus (210^{4}) A. flavus (610^{4}) A. parasiticus (310^{4}) A. niger (210^{4}) F. graminerium (310^{4}) F. verticilloides (610^{4}) Yeast
D13	A. flavus (410^{4}) A. parasiticus (10^{4}) F. graminerium (310^{4}) Penicillium spp (210^{4})
D14	A. fumigatus (10^{4}) A. flavus (310^{4}) A. parasiticus (210^{4}) A. ochraceus (310^{4}) A. niger (210^{4}) F. graminerium (310^{4}) Penicillium spp (110^{4})
D15	A. fumigatus (410^{4}) A. flavus (610^{4}) A. parasiticus (310^{4}) A. niger (210^{4}) Unidentified (310^{4}) Penicillium spp(4) Yeast
D16	A. fumigatus (210^{4}) A. flavus (410^{4}) A. niger (210^{2}) F. graminerium (310^{4}) Penicillium spp(410^{4}) Yeast
D17	A. fumigatus (410^{4}) A. flavus (610^{4}) A. parasiticus (310^{4}) A. niger (210^{4}) F. graminerium (310^{4}) Penicillium spp(410^{4}) Yeast

D18	A. fumigatus (310^4) A. flavus (210^5) A. parasiticus (210^4) A. niger (210^4) A. niveus (210^4) F. graminerium (310^4) F. verticiloides (310^4) Penicillium spp(410^4) Yeast
D19 D20	A. fumigatus (410^4) A. flavus (610^4) A. parasiticus (310^4) A. niger (210^4) A. ochraceus (210^4) A. niveus (310^4) F. graminerium (310^4) Penicillium spp(410^4), Yeast A. fumigatus (610^4) A. flavus (610^4) A. parasiticus (310^4) A. niger (210^4) F. graminerium (310^4) F. verticiloides (610^4) Unidentified (310^4) Penicillium spp(4) Yeast
S32 RABBIT	A. fumigatus (410^4) A. flavus (910^4) A. parasiticus (310^4) A. niger (210^4) F. graminerium (310^4) Penicillium spp(410^4) Yeast Rhizopus spp
S33 MICE	A. fumigatus (310^4) A. flavus (810^4) A. parasiticus (310^4) A. ochraceus (210^4) A. niger (210^4) F. graminerium (310^4) Penicillium spp(410^4) Yeast
S34 RODENT	A. fumigatus (510^4) A. flavus (910^4) A. parasiticus (310^4) A. niger (210^4) F. graminerium (310^4) Penicillium spp(410^4) Yeast

***Key**

C1-12 (Cats)

D1-D20 (Dog)

Appendix 3 – Mycotoxin Analysis of Cat and Dog Samples

Aflatoxins in dog feed samples

	AFB1	AFB2	AFG1	AFG2	TOTAL AFLATOXINSµg/kg
DOG					
1	5.09	10.82	16.47	16.47	**48.85**
2	137.59	16.21	7.60	7.60	**169.00**
3	199.91	21.88	7.81	7.81	**237.41**
4	77.79	7.92	21.60	21.60	**128.92**
5	101.02	11.05	31.00	31.00	**174.05**
6	35.33	11.37	15.49	15.49	**77.68**
7	378.59	30.44	7.50	7.50	**424.04**
8	4.93	1.08	4.58	4.58	**15.17**
9	8.34	3.15	6.20	6.20	**23.90**
10	21.61	3.21	8.71	8.71	**42.24**
11	26.31	1.48	25.21	25.21	**78.22**
12	2.67	3.77	0.68	0.68	**7.80**
13	7.49	2.24	1.58	1.58	**12.89**
14	13.33	3.61	5.73	5.73	**28.39**
15	44.15	1.11	3.24	3.24	**51.74**
16	5.51	1.26	1.29	1.29	**9.35**
17	27.25	3.35	3.80	3.80	**38.20**
18	11.04	3.63	4.78	4.78	**24.23**
19	7.40	2.73	4.10	4.10	**18.34**
20	1.10	0.50	0.66	2.00	**4.26**

Aflatoxins in cat feed samples

CAT	AFB1	**AFB2**	**AFG1**	**AFG2**	**TOTAL AFLATOXINS (ug/ml)**
21	11.61	4.53	0.86	0.86	**17.85**
22	158.87	17.54	1.26	1.26	**178.94**
23	7.66	2.49	1.27	1.27	**12.70**
24	6.28	1.93	0.63	0.63	**9.48**
25	7.90	1.89	0.00	0.00	**9.79**
26	100.24	7.66	3.51	3.51	**114.91**
27	2.20	1.17	1.05	1.05	**5.47**
28	20.09	3.66	8.84	8.84	**41.44**
29	6.96	1.56	0.00	0.00	**8.51**
30	4.66	10.35	6.52	6.52	**28.04**
31	7.47	2.29	3.98	3.98	**17.71**
32	51.53	17.73	12.17	12.17	**93.61**

	Zearalenone	HPLC			
	Area	**ng/g**	**ng/kg**	**ug/kg**	**TLC**
C1	267728.2	91	1824.072	1.8	*
C2	0	0	0	0.0	
C3	79634.7	27	542.5631	0.5	
C4	167449.6	57	1140.859	1.1	*
C5	573349.2	195	3906.314	3.9	*
C6		0	0	0.0	
C7	288620.5	98	1966.415	2.0	**
C8	81767.1	28	557.0915	0.6	
C9	801524.7	273	5460.908	5.5	**
C10	364578.1	124	2483.925	2.5	***
C11	7528316	2565	51291.54	51.3	****
C12		0	0	0.0	
D1	1253046	427	8537.189	8.5	*
D2	0	0	0	0.0	
D3		0	0	0.0	
D4	447678.6	153	3050.101	3.1	*
D5	1614013	550	10996.51	11.0	**
D6	604734.6	206	4120.147	4.1	**
D7	0	0	0	0.0	
D8	0	0	0	0.0	
D9	0	0	0	0.0	
D10	0	0	0	0.0	
D11	0	0	0	0.0	
D12	104906.5	36	714.7437	0.7	*
D13	100446.5	34	684.357	0.7	*
D14	59898.6	20	408.0981	0.4	*
D15	19751.9	7	134.5726	0.1	*
D16	6356.4	2	43.3071	0.0	*
D17	0	0	0	0.0	
D18	0	0	0	0.0	
D19	0	0	0	0.0	
D20	0	00	0	0	
STD1	104576.3	10	ng/ml		
STD2	235501.1	20	ng/ml		
STD3	472570.4	40	ng/ml		

***Key**

C1-C12 (cat)

D1-D20 (dog)

Fumonisins HPLC analysis

Sample	FB1ug/kg	FB2 ug/kg	FB1ug/kg	FB2 ug/kg	Total AFBs ug/kg	TLC
C1	0.60	0.00	2.4	0.0	2.4	
C2	1.38	0.00	5.5	0.0	5.5	*
C3	4.48	0.00	17.9	0.0	17.9	*
C4	26.01	104.16	104.0	416.6	520.7	*
C5	1.28	4.19	5.1	16.7	21.9	*
C6	5.21	17.08	20.9	68.3	89.2	*
C7	1.70	0.00	6.8	0.0	6.8	*
C8	261.78	30.80	1047.1	123.2	1170.3	***
C9	4.74	14.13	19.0	56.5	75.5	*
C10	4.78	7.22	19.1	28.9	48.0	*
C11	20.21	8.17	80.9	32.7	113.5	***
C12	1.19	0.00	4.8	0.0	4.8	*
D1	3.30	5.44	13.2	21.8	34.9	
D2	9.83	14.23	39.3	56.9	96.2	**
D3	6.61	13.18	26.5	52.7	79.2	***
D4	26.11	0.00	104.4	0.0	104.4	**
D5	5.12	0.00	20.5	0.0	20.5	**
D6	40.42	17.10	161.7	68.4	230.1	***
D7	5.11	0.00	20.5	0.0	20.5	*
D8	0.84	0.00	3.4	0.0	3.4	
D9	1.10	0.00	4.4	0.0	4.4	
D10	1.56	0.00	6.3	0.0	6.3	*
D11	3.81	0.00	15.3	0.0	15.3	*
D12	1.76	0.00	7.0	0.0	7.0	**
D13	1.58	5.35	6.3	21.4	27.7	*
D14	6.12	0.00	24.5	0.0	24.5	**
D15	10.80	138.44	43.2	553.8	597.0	**
D16	6.83	6.99	27.3	28.0	55.3	**
D17	2.51	3.62	10.0	14.5	24.5	
D18	24.16	6.99	96.6	28.0	124.6	*
D19	1.07	0.00	4.3	0.0	4.3	
D20	30.50	8012.50	412.00	9610.12	10022.12	***
*Key						
C1-C12 (cat)						
D1-D20 (dog)						

OCHRATOXIN A HPLC analysis

OTA	AREA	ng/ml	ng/25mg	ng/kg	ug/kg	TLC
STD1	155663.6	10				
STD2	351426.9	20				
STD3	660757.8	40				
1	6297.4	0.377678	0.6	30.2	0.03	
2	638381	38.28601	61.3	3062.9	3.06	*
3	432465.9	25.93654	41.5	2074.9	2.07	**
4	44574.8	2.673312	4.3	213.9	0.21	*
5	0	0	0.0	0.0	0.00	
6	59010.1	3.539049	5.7	283.1	0.28	*
7	29949	1.79615	2.9	143.7	0.14	*
8	287198.3	17.22432	27.6	1377.9	1.38	**
9	0	0	0.0	0.0	0.00	
10	540593.4	32.42134	51.9	2593.7	2.59	**
11	186369.6	11.17726	17.9	894.2	0.89	**
12	369426.7	22.15585	35.4	1772.5	1.77	*
13	0	0	0.0	0.0	0.00	
14	42596.3	2.554654	4.1	204.4	0.20	*
15	93373.4	5.59994	9.0	448.0	0.45	*
16	40784.7	2.446006	3.9	195.7	0.20	*
17	26326.6	1.578901	2.5	126.3	0.13	*
18	9283	0.556735	0.9	44.5	0.04	
19	22147.6	1.328272	2.1	106.3	0.11	
20	0	00	0	0	0	
21	55904.3	3.352783	5.4	268.2	0.27	*
22	0	0	0.0	0.0	0.00	
23	42764.8	2.56476	4.1	205.2	0.21	*
24	21659	1.298968	2.1	103.9	0.10	*
25	220615.9	13.23113	21.2	1058.5	1.06	**
26	131884.7	7.909602	12.7	632.8	0.63	*
27	772966.8	46.35761	74.2	3708.6	3.71	**
28	18882.9	1.132476	1.8	90.6	0.09	
29	79659.4	4.777462	7.6	382.2	0.38	*
30	568338.8	34.08533	54.5	2726.8	2.73	**
31	77867.7	4.670007	7.5	373.6	0.37	*
32	10106.5	0.606123	1.0	48.5	0.05	

*Key

C1-12 (Cat)

D13-32 (Dog)

Appendix 4 – Dog Cytotoxic Assays

Dog Cytotoxic Assays

Sample No	EXTRACT	ATP	% Apoptosis	% Necrosis	% Mt Depol	% MTT to Formazan conversion	GSH (uM)	MDA (uM)
39	CONTROL	1246275	7.255	1.825	9.89	102	5.6	0.75
40	CONTROL	1401545	8.545	1.98	8.89	96	5.9	0.88
1	1A	477499.5	12.37	3.76	35.1	98	2.0	0.64
2	2A	599967.5	13.15	3.955	23.85	106	5.3	0.79
3	3A	557023.5	14.1	5.225	25.6	109	6.2	0.75
4	4A	531625.5	12.55	4.145	23.8	107	7.4	0.77
5	5A	348916.5	18.6	6.415	33	94	7.9	0.58
6	6A	433522.5	20.2	7.945	36.2	97	5.3	0.62
7	7A	455096.5	20.3	7.155	33.5	104	2.6	1.00
8	8A	979720.5	17	2.015	11.3	91	7.0	0.92
9	9A	894235	17.15	1.885	10.8	92	7.3	0.88
10	10A	978757.5	17.35	0.86	8.89	105	6.0	0.90
11	11A	888335.5	17.7	1.8	10.45	104	8.2	0.53
12	12A	753269.5	14.15	4.53	18.05	108	5.7	0.83
13	13A	576515.5	16.65	6.48	26.75	112	2.5	0.68
14	14A	974111	14.7	2.275	12.4	115	5.5	0.85
15	15A	875803.5	7.175	1.995	11.2	102	3.2	0.64
16	16A	1090740	17.3	1.665	9.955	86	3.8	0.94
17	17A	788.7995	1.99	97.7	99.45	75	3.2	0.77
18	18A	779615.5	12.5	4.575	10.7	116	2.0	0.77
19	19A	483387	21.7	9.175	35.1	112	-0.4	0.60
20	1N	515314.5	10.095	3.64	10.955	97	4.5	0.79
21	2N	769729	10.1	3.21	10.9	119	3.9	0.64
22	3N	795747	11.55	3.195	11.05	115	3.6	0.68
23	4N	629190	11.3	4.41	22.25	97	3.7	1.28
24	5N	346744	7.525	6.37	36.45	86	1.7	0.96
25	6N	407121	14.25	8.635	37.35	97	1.6	0.79
26	7N	655307	12.15	5.31	19.55	105	6.0	0.88
27	8N	333334	4.265	15.45	41.9	106	5.5	0.88
28	9N	607.077	7.41	13.9	41.2	78	6.3	0.79
29	10N	685205.5	15.3	4.9	24.35	109	5.7	0.81
30	11N	701688.5	15.2	5.975	18.25	111	7.3	0.94
31	12N	676355.5	14.7	7.995	19.5	102	2.0	0.83
32	13N	975214.5	12.3	3.185	12.05	97	3.1	0.94
33	14N	967517.5	15.15	3.465	15.95	95	3.0	0.83
34	15N	1022065	14.4	2.565	12.35	99	2.6	0.79
35	16N	1094205	17.55	2.44	12	97	0.7	0.94

36	17N	792109	12.45	4.895	16.05	101	0.5	0.90
37	18N	479751	11.35	7.82	18.95	89	1.0	0.85
38	19N	1130530	20.15	3.075	8.525	100	5.2	0.90
41	1AN	1337598.5	10.35	2.135	8.42	82	5.6	0.77
42	2AN	884235	10.7	3.965	8.435	109	3.5	0.77
43	3AN	616267.5	16.55	7.245	23.2	111	1.9	0.83
44	4AN	748768.5	11.75	4.43	15.965	105	7.0	0.92
45	5AN	784830	13.25	5.58	11.1	109	6.8	0.94
46	6AN	899519.5	13.7	5.385	9.62	105	6.9	0.77
47	7AN	843753.5	15.35	6.315	9.19	99	0.1	0.88
48	8AN	483270.5	13.3	10.55	42.2	94	4.7	0.94
49	9AN	325637	9.34	6.555	17.4	86	0.6	0.96
50	10AN	640755.5	14.35	4.79	10.6	114	3.8	0.83
51	11AN	776182.5	15.4	4.41	10.55	116	4.8	1.11
53	12AN	653552	17.1	5.075	9.09	113	6.5	0.79
54	13AN	721242	16.9	4.895	9.68	113	5.6	0.92
55	14AN	704472	15.65	6.155	19.05	108	0.8	3.23
56	15AN	668151	19	8.455	28.45	100	1.1	0.85
57	16AN	446873.5	21	4.065	10.125	89	3.6	0.90
58	17AN	398713	21.6	3.92	11.05	133	2.9	0.96
59	18AN	788951	17.95	3.745	15.35	96	-0.2	0.82
60	19AN	901368	22.8	2.6	13.55	100	2.6	1.88

Dog Caspase Assay

Sample No.	EXTRACT	CASPASE 3/7 ACTIVITY	CASPASE 8 ACTIVITY	CASPASE 9 ACTIVITY
39	CONTROL	1322845	937185	203290.5
40	CONTROL	1089480	757520	162446
1	1A	1273385	786790	177150.5
2	2A	1318955	762821.5	207635
3	3A	1427350	803031.5	228207.5
4	4A	1303365	665258.5	227576
5	5A	1360090	731059.5	221369
6	6A	1405810	655827.5	228201.5
7	7A	1432790	675700.5	224729.5
8	8A	1440515	1026450	272960
9	9A	1079110	979338.5	219390.5
10	10A	1278005	1001921	213554.5
11	11A	1260585	995588	237917
12	12A	1310645	1024475	241133.5
13	13A	1359015	806335.5	221123.5
14	14A	1264520	1121435	252755
15	15A	1266560	969493.5	233049
16	16A	1378000	1050065	260953
17	17A	55843.4	337576	163791
18	18A	1301640	943802.5	212268
19	19A	1490735	709747.5	207776
20	1N	1312335	936359.5	228339
21	2N	1378605	885255.5	251086
22	3N	1349055	954253	243505
23	4N	1376135	888803	254130.5
24	5N	1550830	869240	235719
25	6N	1212780	606820	160098
26	7N	1371865	803094	210731
27	8N	1453285	841643.5	239130
28	9N	75527.85	11006.8	188608.5
29	10N	1390210	676573	349639
30	11N	1398805	937212.5	239237.5
31	12N	1393135	883520.5	225849.5
32	13N	1163810	1012775	205101
33	14N	1217805	964759	187678.5
34	15N	1258855	1022890	220787
35	16N	1220780	1040228	226359
36	17N	1323535	1014195	232129
37	18N	1286730	884675.5	222586.5
38	19N	1333410	1132265	248618
41	1AN	1105195	783081	180891
42	2AN	1326180	1009570	231632
43	3AN	1337175	904520.5	206437.5
44	4AN	1237685	977766.5	219538
45	5AN	1390370	965817.5	243446
46	6AN	1358450	997185	227916.5
47	7AN	1401475	900709	235060
48	8AN	1482055	886492	256086

49	9AN	1232190	693987.5	187188
50	10AN	1340245	780040	217879.5
51	11AN	1200770	830639.5	215783.5
53	12AN	1170890	716209.5	198044.5
54	13AN	1201330	833629	213881.5
55	14AN	1252680	818325.5	218958
56	15AN	1224120	800553	171170
57	16AN	1348070	639296	162621.5
58	17AN	1201255	627826	192817
59	18AN	1169390	783438.5	193328.5
60	19AN	1104440	681539	199898

Appendix 5 – Cat cytotoxic assays

Sample No.	EXTRACT	ATP	% Apoptosis	% Necrosis	% Mt Depol	GSH (uM)	MDA (uM)
Control	c	487262	14	2	27	2.2	0.09
Control	c	462321	13.5	2.1	25	2.1	0.11
Control	c	492832	14.2	1.89	28	2.4	0.08
1	a	239748	62	6	39	1.09	0.27
2	a	2542	27	72	5	0.69	0.35
3	a	2376	2	98	1	0.19	0.55
4	a	311530	53	27	31	2.23	0.46
5	a	3969	5	85	22	0.53	0.68
7	a	57587	12	78	35	1.76	0.80
8	a	3301	21	79	2	0.74	0.66
9	a	649539	1	13	20		0.78
10	a	2014	0	1	78	0.18	0.26
11	a	2344	0	18	7	0.49	0.45
12	a	4311	31	24	12	1.91	3.06
	Average	**194405**	**18**	**36**	**24**	**0.97**	**0.62**
13	n	2572	52	28	33	0.82	0.51
14	n	99745	10	36	68	0.04	0.15
15	n	3985	23	60	51	0.90	0.27
16	n	217804	8	27	68	1.59	0.54
17	n	106455	10	34	69	1.10	0.30
19	n	163286	15	25	68	1.21	0.16
20	n	457758	18	20	67	0.43	0.21
21	n	2380				1.87	0.20
22	n	5077	30	62	19	0.12	0.25
23	n	3637	65	30	49	0.13	0.26
24	n	8820	16	63	59	1.31	0.27
	Average	**97411**	**25**	**38**	**55**	**0.87**	**0.28**
25	an	3279	1	1	35	1.66	0.49
26	an	4791	46	39	32	1.19	0.18
27	an	2490	68	30	1	1.58	0.17
28	an	263503	24	13	53	1.80	0.31
29	an	15898	39	41	44	1.09	0.19
31	an	210673	39	24	52	0.17	0.26
32	an	10816	53	37	27	0.16	0.18
33	an	4099	2	31	4	0.01	0.27
34	an	2776	0	5	42	0.53	0.21
35	an	2420	24	45	12	0.19	0.35
36	an	4019	58	40	18	1.63	0.36
	Average	**47706**	**32**	**28**	**29**	**0.9**	**0.27**

Cats Caspase Assay

Sample No.		EXTRACT	CASPASE 3/7 ACTIVITY	CASPASE 8 ACTIVITY	CASPASE 9 ACTIVITY
Control		c	13228	93718	20329
Control		c	13726	89273	21927
Control		c	14192	91827	19827
1		a	1266860	48368	44621
2		a	44881	4833	6930
3		a	1934	2902	5323
4		a	961594	15666	43872
5		a	132749	3783	4729
7		a	623143	5313	19414
8		a	96305	6404	9773740
9		a	200756	27051	647666
10		a	788	3897	1684
11		a	3409	11835	3409
12		a	357168	7461	4337
		Average	**266481**	**29452**	**758415**
13		n	2916	1126	1660
14		n	261891	5297	13383
15		n	134235	4241	5391
16		n	844287	9536	23278
17		n	332547	5371	9192
19		n	673043	4503	16874
20		n	1214700	8391	24601
21		n	1480	7098	1872
22		n	77068	2840	2792
23		n	1162	5541	1188
24		n	170858	420	4931
		Average	**337653**	**4942**	**9560**
25		an	156034	5579	6822
26		an	174933	4151	6632
27		an	10922	3305	4721
28		an	860537	15039	32188
29		an	212453	3737	3707
31		an	526372	5395	13899
32		an	306053	2800	5900
33		an	6914	6092	1604
34		an	750	33191	2058
35		an	1688	8051	2288
36		an	305769	382	1608
		Average	**232948**	**7975**	**7402**