MYCOLOGICAL QUALITY AND AFLATOXIN M1 CONTAMINATION OF MILK AND MILK PRODUCTS FROM BOMET COUNTY, KENYA

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Mycological Quality and Aflatoxin M1 Contamination of Milk and Milk Products from Bomet County, Kenya

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DECLARATION

This thesis is my original work and it has not been presented for a degree in any other university.

Signature: …….. Date: ……

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This thesis has been submitted for examination with our approval as university supervisors.

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DEDICATION

I dedicate this work to my family, for their patience, moral and financial support throughout the study period.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

AC	Activated carbon
AFs	Aflatoxins
AFB1	Aflatoxin B1
AFM1	Aflatoxin M1
ANOVA	Analysis of Variance
CFU	Colony forming units
EEC	European Economic Community
ELISA	Enzyme Linked Immunosorbent Assay
EU	European Union
FAO	Food and Agriculture Organization
FDA	Food and Drugs Act
HPLC	High performance liquid chromatography
IARC	International Agency for Research on Cancer
KEMRI	Kenya Medical Research Institute
LPCB	Lacto Phenol Cotton Blue
Ppb	Parts per billion
Rpm	Revolutions per minute
SDA	Sabouraud Dextrose Agar
TLC	Thin layer chromatography
UHT	Ultra Heat Treated
W.H.O	World Health Organization

ABSTRACT

Milk and dairy products form essential constituents in daily meals of the residents of Bomet and other parts of Kenya. Dairy products are highly nutritious and may be susceptible to contamination by bacteria, molds and/or mycotoxins, and in particular aflatoxin B1 which is the most toxic. Aflatoxin M1 (AFM1) is a hepatic carcinogenic metabolite of aflatoxin B1 (AFB1) found in the milk or milk products obtained from livestock that have ingested feeds contaminated with AFB1. The aim of this study was to determine the mycological quality and aflatoxin M1 contamination of milk and its products from Bomet County, Kenya. A total of two hundred and twenty seven; (one hundred and ninety two raw and thirty five processed milk samples) were purchased from shops, milk vendors, kiosks and milk collection centers respectively. Isolation was done on Sabouraud Dextrose Agar (SDA) and identification was done using macro and micromorphological features and confirmed using Analytical profile index (API 20 C aux). Determination of aflatoxin M1 contamination was by direct competitive ELISA technique. Three hundred and sixty five fungal isolates of which 27(7.4%) were molds and 338(92.6%) yeasts were isolated from all the raw milk samples examined. Processed milk and its products showed no contamination with yeasts and molds. The isolated fungi belonged to the genera *Aspergillus* 3% (11/27), *Geotrichum* 4.1% (15/27) and *Fusarium* 0.3% (1/27), *Candida* 57.8% (211/365), *Saccharomyces* 6.6% (24/365), *Cryptococcus* 1.1% (4/365) and *Rhodotorula* 27.1% (99/365). Analysis of the data indicated that the overall occurrence of aflatoxin M1 contamination above the threshold limit of 0.05 ppb by Food and Agriculture Organization (FAO) and World Health Organization (WHO) limits was 43.8% (81/185). This was contributed mainly by the raw milk samples which had 52% (78/150) AFM1 contamination compared to processed milk with 8.6% (3/35). The difference was statistically significant, $p<0.0001$. The overall median was 0.02 (IQR: 0.00, 0.40) $\mu g/l$ which is below the threshold limits of 0.05 ppb by FAO/WHO. The levels of AFM1 in milk samples indicate that the feeds given to dairy cows in the study area could be contaminated with aflatoxin B1 which is the precursor of aflatoxin M1. This poses a potential risk of chronic exposure to the

residents and therefore there is need for regular monitoring of contamination in milk and control most contaminating causes with special focus on occurrence of AFB1 in the feed of dairy cows. There is also need for awareness creation on aflatoxins in the to sensitize people on health hazards associated with aflatoxin M1 contamination as there may be chronic mycotoxin exposure.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Milk and dairy products have been used by humans since prehistoric times, as one of the most popular and nutritious human food. This value put the food hygienists in a real challenge, to provide safe milk to consumers while maintaining its nutritional value. It is estimated that billions of people around the world consume milk and dairy products every day as they are the vital source of nutrition for human health. Milk for human consumption is mostly derived from cows but milk from other animals such as; buffaloes, goats, camels and sheep are also used for the production of dairy products. According to Bomet county data, the production of milk per day per animal is 4.3 liters, leading to a yearly production of 201,639,314 liters.

Fungal contamination of dairy products can occur from the environment, equipment, milk handlers and packaging materials. Molds and yeasts are recognized as an important cause of spoilage of various dairy products (Khalifa *et al.,* 2013; Pal & Jadhav, 2013; Pal *et al.,* 2014). *Aspergillus, Fusarium* and *Penicillium* are of public health concern as these fungi are known to produce mycotoxins that are hazardous to human health (Khalifa *et al.*, 2013; Sengum *et al.,* 2008). Most of the fungi isolated from milk and dairy products include: *Candida glabrata, C. guillermondii, C. tropicalis, Cryptococcus albidus, Cr. laurentii, Cr. luteolus, Debaryomyces hansenii, Geotrichum candidum, Hansenula polymorpha, Kluyeromyces bulgaricus, K. lactis, K. maxianus, Mucor racemosus, Pichia*

fermentans, Rhodotorula glutinous, Saccharomyces cerevisiae, Trichosporon asahii and *Yarrowia lipolytica* (Pal, 2007).

Mycotoxins are fungal secondary metabolites which when ingested cause a variety of adverse effects on both humans and animals (Hampikyan *et al*., 2010). Aflatoxins are toxic by-products produced predominantly by two filamentous fungi; *Aspergillus flavus* and *Aspergillus parasiticus* (Baskaya *et al.,* 2006). *Aspergillus* species are capable of growing on a diversity of substrates under varied environmental conditions mainly in tropical and subtropical climates. Aflatoxins therefore, occur as natural contaminants in many agricultural commodities produced during growth, harvesting and storage (Kensler *et al.,* 2011; Prandini *et al.,* 2009). There are more than 20 known aflatoxins, but the four main ones are aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) (Inan *et al.,* 2007). Aflatoxin M1 (AFM1) and M2 (AFM2) are the hydroxylated metabolites of AFB1 and AFB2 (Giray *et al.,* 2007). Predominant species with aflatoxin production ability include *Aspergillus flavus* and *A. parasiticus* (Yu *et al.,* 2004). Other aflatoxin producing species include *Aspergillus nomius, A. pseudotamarii, A. bombycis, A. toxicarius, A. parvisclerotigenus, A. ochraceoroseus, A. rambellii* or the ascomycete genus *Emericella* by *Emericella astellata* and *E. venezuelensis* (Frisvad *et al.,* 2004; Frisvad *et al.,* 2005; Ito *et al.,* 2001; Kurtzman *et al.,* 1987; Peterson *et al.,* 2001; Reiter *et al.,* 2009). Most studied aflatoxin is aflatoxin B1 as it is identified to be most toxic and potentially hepatocarcinogenic (Bennett & Klich, 2003).

Aflatoxin M1 (AFM1), known as milk toxin, is the principle hydroxylated product of AFB1, metabolized by cytochrome P450 associated enzymes in liver. It appears in milk, feces and urine of lactating animals following consumption of the AFB1 contaminated feeds (Aycicek *et al*., 2005; Fallah *et al*., 2009). Milk, a natural liquid and one of most nutritionally complete foods widely used in many countries, is considered a significant risk for AFM1 exposure in human (Fallah, 2010; Ghazani, 2009). Many studies have reported the occurrence of high levels of AFM1 in milk that exceeded the maximum allowed limits of 0.05 parts per billion (Amer & Ibrahim, 2010; Dashti *et al*., 2009; Hussain & Anwar, 2008; Kamkar *et al.*, 2011; Panahi *et al.*, 2011; Tsakiris *et al.*, 2013). According to a study carried out to determine the level of aflatoxins in milk from urban smallholder farmers in Nairobi, Nakuru, Nyeri, Eldoret and Machakos, thirty five percent of the positive samples had aflatoxin levels exceeding 0.05ppb; the FAO/WHO and EU acceptable level of aflatoxin M1 and aflatoxin M2 for milk (FAO, 1990; FAO, 1992; Kang'ethe & Lang'at 2009). Aflatoxin M1 has been detected in milk within 12-24h after the first ingestion of AFB1 in feeds and its concentration decreased to undetectable levels within 72 hours. The amount excreted as AFM1, as a percentage of AFB1 in feed, is usually 1-3% (Azizollahi *et al.*, 2012).

The risk posed by aflatoxins has been recognized in different countries. In Europe, the maximum tolerated levels of AFM1 in milk and dairy products were first regulated by Regulation CE 2174/2003 that modified Regulation CE 466/2001, and then by Regulation 1881/2006 (Official Journal of European Communities, 2001; Official Journal of European Communities, 2003; Official Journal of European Communities,

2006). In these regulations, AFM1 concentration in milk must not exceed 0.05ppb and dairy products obtained from milk must conform to the above AFM1 limits. Kenya has adopted the Food and Agriculture Organization (FAO) and World Health Organization (WHO) limit of 10ppb total aflatoxins and 5ppb AFB1 in food. Despite having established these regulations, they are poorly enforced especially for foods that pass through informal markets, from where majority of Kenyans get their supply, thus putting them at a high risk of exposure. This study was therefore done to determine the mycological quality and presence of AFM1 in milk and its products in Bomet County.

1.2 Statement of the problem

Mycotoxins especially aflatoxins in cattle feed and their consequential presence in milk is a serious concern globally due to their potent carcinogenicity. Several researchers have reported of potential hazardous human exposure to AFM1 through milk. The World Health Organization (WHO) does not recognize aflatoxins as a high-priority problem as measured by disability-adjusted life years in their analysis of factors contributing to the burden of disease across the world. Unfortunately, the rising cases of cancer are of great concern. Bomet County, Kenya has the highest rates of esophageal cancer accounting for 34.6% of the newly diagnosed cancers and factors associated with these high incidences are not clear. This is according to a study done at Tenwek Hospital (Bomet County), where cases were analyzed within and outside a traditional catchment area defined as ≤ 50 km from the hospital (Parker *et al.,* 2010). Although AFM1 has not been linked directly with esophageal cancer, other fungal metabolites such fumonisins and aflatoxins (Marasas, 2001; Wild & Gong, 2010) have been associated with esophageal and liver

cancer respectively. The causes of cancer are multifactorial and AFM1 exposure could be a risk factor.

Bomet is one of the leading counties that practice dairy farming. Exposure to high levels of AFM1 in milk is a possibility since the community relies mainly on milk and its products for nutrition but public awareness of mycotoxins is not currently available.

1.3 Justification

Dietary intake is the main route through which humans as well as animals are exposed to aflatoxins. Due to the widespread consumption of milk, presence of aflatoxin M1 (metabolite of AFB1) is a worldwide concern. The carcinogenic and highly toxic effects of aflatoxin and its metabolites have resulted in aflatoxin being highly regulated by most countries in the world. Once it exceeds the regulatory limits, the AFM1 contaminated milk, by law, has to be discarded to prevent it from getting back into the food chain. The daily intake of contaminated milk especially in children leads to a build-up of aflatoxins with serious health consequences later in life. This study sought to determine the mycological quality and aflatoxin M1 contamination of milk and its products in Bomet County. The county is in a rural setup and the community being pastoralist depend highly on milk for nutritional requirements. This information is vital for enacting measures aimed at reducing mycotoxin exposure through daily dietary intake of milk.

1.4 Objectives

1.4.1 General objective

1. To determine the fungal contaminants and levels of aflatoxin M1 in milk and its products from Bomet County in Kenya.

1.4.2 Specific objectives

1. To determine and identify the fungal contaminants in milk and its products from Bomet County.
2. To determine the occurrence and aflatoxin M1 levels in milk and its products from Bomet County.
3. To compare the fungal contaminants mycological quality and aflatoxin M1 levels in processed and unprocessed milk samples from Bomet County.

CHAPTER TWO

LITERATURE REVIEW

2.1 Mycotoxins

Mycotoxins are toxic fungal secondary metabolites produced by fungi (molds). These fungal metabolites are chemically diverse with varying molecular weights. There are hundreds of mycotoxins known, but few have been extensively researched and even fewer have good methods of analysis available. The fungal metabolites are produced by fungi on crops in the field, during transport, handling and in storage. Mycotoxin ingestion can lead to illness in humans with symptoms such as nausea, vomiting, diarrhea and headache (Creppy, 2002), while some mycotoxins have carcinogenic potential (Murphy *et al*., 2006). Although the potentially harmful effects of feeding on moldy grain and foods has been known for many years the term mycotoxin was coined in 1962 in the aftermath of an unusual veterinary crisis near London, England, during which approximately 100,000 turkeys died from a mysterious Turkey X disease. The outbreak was linked to peanut (groundnut) meal imported from Brazil (Sargeant *et al*., 1961). Because of intensive multidisciplinary research efforts, a blue-fluorescent toxin was detected and was associated with the isolation of *Aspergillus flavus* (Bennett & Klich, 2003). The fungus was soon shown to produce the same toxic compound(s) found in the toxic peanut meal. The toxin was characterized chemically and biologically and was given the name, aflatoxin. The most important genera of food mycotoxigenic fungi are *Aspergillus, Fusarium* and *Penicillium*. Examples of mycotoxins of greatest public

health and agro-economic significance include aflatoxins, trichothecenes, zearalenone, fumonisins and ochratoxins.

2.2 Milk Microbial Contaminants

Milk, besides being a nutritious food for humans, provides a favorable environment for the growth of microorganisms. Once microorganisms get into the milk their numbers increase rapidly and bring about spoilage making raw or processed milk unsuitable for human consumption due to rancidity, musty odors, or toxin production (Nanu *et al.*, 2007). The presence of these microorganisms usually indicates inadequate collection (milking) procedures, poor storage conditions, unhygienic production and human factors (Gran *et al.*, 2003; Grimaud *et al.*, 2009).

Presently, over 250,000 fungi are present in our environment. They are ubiquitous in nature, and are found in the soil, water, and air. Fungi have great importance in the technological processes of making dairy products and they can be used to judge milk sanitary quality and the conditions of dairy production (Delavenne *et al.*, 2011; Spanamberg *et al.*, 2004). The type of spoilage fungi differ widely among dairy products because of the effects of practices followed in the production, formulation, processing, packaging, storage, distribution and handling. Warm climate and inadequate refrigeration are the principal causes of high level of contamination due to fungi. Some physical defects such as off color, loss of firmness and loss of aroma can occur following the spoilage of milk products by fungi.

The common examples of yeast genera frequently isolated from milk include *Candida, Debaromyces, Hansenula, Kluveromyces, Pichia, Rhodotorula, Saccharomyces* and *Trichosporon* (Pal, 2007). Yeast spoilage is particularly important in fermented milk products and cheeses, and less in fresh or pasteurized milk, cream and butter. In fruit yoghurts, yeasts may be introduced by non-dairy ingredients such as fruits, sugar, honey and nuts (Fleet, 1990; Jakobsen & Narvhus, 1996). Yeasts can play a major role in dairy fermentations due to a number of their physiological and biochemical characteristics, including the ability to utilize lactose or galactose, for example, in *Debaromyces hansenii*; high proteolytic or lipolytic activity, for example, in *Yarrowia lipolytica* and *Geotrichum candidum*; ability to grow at low temperatures and tolerance to high salt concentrations (Sacristán *et al*., 2012; Van den Tempel & Jakobsen, 2000). More generally, spoilage yeasts can be introduced during the entire production chain, ranging from the farm, dairy plant, to the final product. Hygiene and sanitation measures are important to control contamination of dairy products with yeasts (Guerzoni *et al.*, 1998).

Molds can be found in milk as contaminants from the environment but they are important in the manufacture of cheese and other dairy products. Molds have the ability to enhance the flavor and aroma and modify the texture and structure of milk-derived products as a consequence of extensive proteolysis and lipolysis. The mold genera that are most commonly detected in raw milk include *Penicilium, Geotrichum, Aspergillus, Mucor* and *Fusarium* (Lavoie *et al*., 2012). At the species level, *Fusarium merismoides, Penicilium glabrum, Penicilium roqueforti, Aspergillus fumigatus, Engyodontium album*,

as well as species of *Cladosporium* and *Torrubiella* are common (Delavenne *et al*., 2011). Although molds are not expected to survive the pasteurization and sterilization treatments applied during milk processing, their spores can tolerate harsh environmental conditions but are sensitive to heat treatment. Their presence in raw milk is undesirable because their enzymatic activities may alter milk constituents and affect organoleptic characteristics of dairy products, produce mycotoxins, and represent a potential health risk (Torkar & Teger, 2008; Torkar & Vengust, 2008).

2.3 Aflatoxins and AFM1 contamination

Aflatoxins are natural toxic compounds produced mainly as secondary metabolites by *Aspergillus flavus* and *Aspergillus parasiticus*. Their growth is influenced by various factors like temperature, relative humidity, oxygen availability, and damaged or broken grain kernels (Awasthi *et al.,* 2012). They are extremely toxic, immunosuppressive, carcinogenic, mutagenic and teratogenic substances known to induce hepatic carcinogenesis in humans. Aflatoxin B1 (AFB1) is the most common and has been reported as the most powerful natural carcinogen in human and animals (Hussain *et al.,* 2008; Tokar & Vengust, 2008). When AFB1 is consumed in feeds it is degraded by rumen to AFM1 within 12-24 hours. Aflatoxin M1 (AFM1) is a hydroxylated metabolite of aflatoxin B1 and is secreted in milk of dairy cattle after consumption of feed contaminated with aflatoxin B1 (Dashti *et al.,* 2009; Fallah, 2010; Iha *et al.,* 2011).

Contamination can occur either by ingestion of food contaminated with aflatoxin or ingestion of aflatoxins in animal feed carried in milk (Agag, 2004), or by inhaling dust

of aflatoxins in contaminated food industries and factories (Ali *et al.,* 2013). Studies have shown that ruminants have little protection against this toxin (Jouany & Diaz, 2005). Consumption of milk may be the principle way for entrance of AFM1 into the human body (Galvano *et al*., 2001).

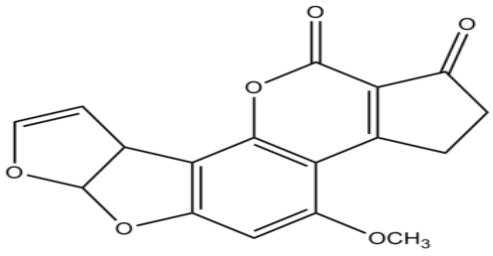
Analysis of aflatoxin levels in 319 raw milk samples from 14 regions in Iran showed that 54% were contaminated with AFM1 with 33% of the samples having levels above the recommended limit of 0.05 ppb (Tajkarimi *et al.,* 2007). It has been demonstrated that up to 6% of the ingested AFB1 is secreted into the milk as aflatoxin M1 (Van Egmond & Dragacci, 2001). Aflatoxin M1 contamination of milk and cheese samples in Africa, is as high as 71.4 % with concentrations of AFM1 ranging from 0.03 to 3.13ppb whereas the maximum tolerance limit is 0.05ppb (Elgebri *et al.*, 2004). The percentage of AFM1 contamination that has been reported in Sudan is as high as 95.45% with contamination level ranging between 0.22 and 6.9 ppb and average concentration of 2.07 ppb greater than the maximum tolerance limit (0.05 ppb) (Elzupir & Elhussein, 2010).

2.4 Chemistry and metabolism of aflatoxin M1

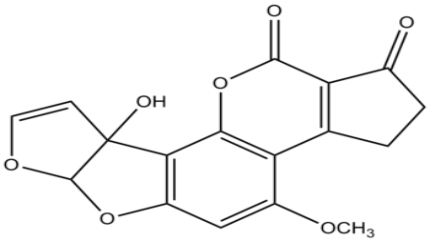
Aflatoxins are highly liposoluble compounds and are readily absorbed from the site of exposure usually through the gastrointestinal tract and respiratory tract into blood stream (Agag, 2004; Larsson & Tjalve, 2000). They are then distributed in blood to different tissues and to the liver, the main organ of metabolism of xenobiotics. Aflatoxins are metabolized by enzymes in the liver to a reactive epoxide intermediate or hydroxylated to aflatoxin M1 (Wild & Montesano, 2009; Wu & Khlangwiset, 2010). Aflatoxin M1

represents the 95% of aflatoxins detected in milk. Other metabolites, such as M2 (AFM2), aflatoxicol (AFL), M4 (AFM4), and Q1 (AFQ1), are detected in trace amounts and, thus, considered of less significance for public health (EFSA, 2004).

Aflatoxin M1 (AFM1) is a hepatocarcinogen 4-hydroxylated derivative of aflatoxin B1 (AFB1), which has a relative molecular mass of 328 daltons and has the molecular formula C17H12O7 (Wagacha & Muthomi, 2008). It is excreted into the milk in the mammary glands of both human and lactating animals that have been fed with AFB1 contaminated diet (Gurbay *et al.*, 2010). It can also be detected in urine, blood, and internal organs. The level of converted AFB1 into AFM1 in milk is influenced by many factors including breed of the animal, health, type of diet, milk production and rate of digestion (Duarte *et al.*, 2013).



Aflatoxin B1



Aflatoxin M1

Figure 2-1: Chemical structures of aflatoxin B1 and M1 (AFM_1) (IARC, 1993)

Studies of AFM1 metabolism have shown that the rate between the amount of AFB1 ingested by cows and the quantity excreted in milk is usually 0.2 to 4% (Henry *et al.*, 2001; Sassahara & Yanaka, 2005).

2.5 Stability of aflatoxin M1 in milk and dairy products

Milk is a highly perishable product that rapidly loses its homogeneity and spoils if untreated. There have been conflicting results from the effect of thermal treatments on AFM1 reduction in milk. Some studies indicate that heat does not cause an appreciable change in the amount of AFM1 in milk whereas others report different levels of decontamination. According to a previous study, sterilization of milk at 121 °C for 15 minutes caused 12.21% degradation of AFM1, whereas boiling decreased AFM1 by 14.50% (Deveci, 2007). A previous study revealed reductions of up to 32% in AFM1 during heat treatments, while others have indicated that AFM1 is heat stable (Galvano *et al.,* 1996; Kabak, 2012). There are reports however, that aflatoxin-contaminated food tolerates the thermal inactivation, pasteurization, autoclaving and other food processing procedures (Kav *et al.*, 2011).

The stability of AFM1 contaminated whey and deproteinized whey subjected to different technological treatments was observed (Cattaneo *et al.,* 2013). During ricotta cheese production, the majority of AFM1, 94% on average, was removed in the discarded whey, only 6% remained in the curd. The use of ultrafiltration and diafiltration removed more than 90% of the toxin remaining in the whey or deproteinized whey discarded from ricotta cheese production. Spray-drying was efficient in reducing AFM1 contamination in whey, where toxin retention was approximately 60%, while in deproteinized whey, the AFM1 retention was approximately 39%. A previous study found that AFM1 was stable in kashar cheese for over 60 days and in traditional white pickled cheese for over 90 days (Oruc *et al.,* 2006). A study was done to observe the stability of AFM1 in

yoghurt artificially contaminated with concentrations of 0.05 and 0.1μg/L during storage for 4 weeks at 4ºC and at pH values of 4.0 and 4.6 (Govaris *et al.*, 2002). It was observed that at pH 4.6, the AFM1 levels did not significantly change ($p > 0.01$); however, in the yoghurt at pH 4.0, AFM1 decreased significantly ($p < 0.01$) after the third and fourth weeks of storage at both concentrations.

In a similar study, during the fermentation of yoghurt, the AFM1 levels decreased significantly ($p < 0.01$) from the initial levels present in milk. Another study investigated the binding ability of AFM1 by Lactic acid bacteria (LAB) such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus* and found that they were effective in reducing free AFM1 content in liquid culture medium and during yogurt processing (El Khoury *et al*., 2011). Researchers have however mentioned that reduced recovery of AFM1 may not mean reduced toxicity and avoiding contamination appears to be the only practical and economical way to ensure safety of milk products for human.

2.6 Toxicity and Health implications of AFM1

It is estimated that about 35% of human cancers are directly related to diet, and the presence of aflatoxins in foods is considered an important factor in the formation of liver cancer, mainly in tropical countries characterized by high moisture content and high temperatures that favor fungal growth. According to WHO, global hepatocellular carcinoma is the leading cause of cancer death (WHO, 2008). Each year 550,000–600,000 new cases of hepatocellular carcinoma are diagnosed, of which Sub Saharan Africa and East Asia contributes eighty three percent of deaths (Kirk *et al*., 2006). Evidence of acute aflatoxicosis in humans has been reported worldwide especially in the

third world countries like Taiwan, Uganda, India, Kenya and many others. The chronic primary aflatoxicosis results from ingestion of low to moderate levels of aflatoxins (USAID, 2012). Some of the common symptoms are impaired food conversion and slower rates of growth with or without the production of an overt aflatoxin syndrome (WHO, 2000). The toxic activity of AFs is due to their capacity to interact with nucleic acids, nucleoproteins and protein syntheses. Aflatoxin M1 has been known to have cytotoxic, genotoxic, and carcinogenic effects (Awad *et al.,* 2012; Fallah 2010). Its carcinogenicity has thus been reclassified by the International Agency for Research on Cancer (IARC) of World Health Organization (WHO) to be group 1 instead of group 2 (Food Safety Watch, 2012; IARC, 2002). Aflatoxin M1 has been demonstrated to be cytotoxic on human hepatocytes *in vitro* and its acute toxicity in several species is similar to that of aflatoxin B1.

Aflatoxin M1 also exhibits a high level of genotoxic activity and certainly represents a health risk because of its possible accumulation and linkage to DNA (Makun *et al*., 2012; Shundo & Sabino, 2006). Moreover, AFB1 contamination at higher levels has also been correlated with reduced birth weight and jaundice in neonates (Abulu *et al*., 1998). The capacity of biotransformation of carcinogens in infants is generally slower than that of adults, resulting in a longer circulation time of toxicity (Sadeghi *et al*., 2009). Studies have shown that in Kenya, young children are introduced to cow's milk at an early age (Bwibo & Neumann, 2003) and therefore consumption of milk contaminated with AFM1 may reduce the development of their immune competence making them more susceptible to other diseases.

2.7 Methods for aflatoxin determination

Several methods of detection have been used or developed for detection of AFM1 in milk and dairy products in the past decade. The available methods for aflatoxin determination include: High Performance Liquid Chromatography (HPLC), Thin Layer Chromatography (TLC), Gas Chromatography (GC), Liquid Chromatography/Mass Spectrometry (LC/MS), Enzyme-Linked Immunosorbent Assay (ELISA) and rapid tests (Pascale & Visconti 2008). Verification of these methods has been done by Association of Analytical Chemists International (AOAC) and by various international committees (AOAC, 2000; IARC, 1993). The tests vary and depend on various factors such as cost effectiveness, precision, and the number of samples to be analyzed. Among screening methods is the enzyme- linked immunosorbent assay (ELISA) that has been adopted widely for screening of AFM1 because of its simplicity, speed, cost effectiveness, adaptability and sensitivity (ICRISAT, 2007). A previous study showed that ELISA method (Ridascreen AFM1) has good results in comparison to high performance liquid chromatography (HPLC) for determination of AFM1 in milk (Rosi *et al.,* 2007). It allows for analysis of multiple samples which is ideal for screening purposes. A review of ELISA as a method for the detection of mycotoxins in milk and dairy products indicated that up to date, it has been applied mainly for AFM1 (Ridascreen) (Enne *et al.,* 2005; Moatsou & Anifantakis, 2003). Fast AFM1, produced by R-Biopharm (Germany) has been very frequently used in several studies (Chen *et al.,* 2004). The High Performance Liquid Chromatography (HPLC) is ideal for validation and quantification as it is highly sensitive, has good selectivity and is easily automated. However, HPLC's

disadvantage is the high cost, making it unsuitable for routine procedures (Trucksess, 1998).

2.8 Legislation and control

The death of 100,000 turkeys in Great Britain in the 1960s as a result of aflatoxin exposure from their feed posed a great concern about the potential hazards by dietary aflatoxins. When it became evident that aflatoxin exposure caused cancer in many species, most countries, established various regulations for aflatoxin levels in food and/or feed in order to limit exposure to this group of mycotoxins (Van-Egmond *et al*., 2007). These initial regulations on aflatoxins were not based on the derivation of a TDI (estimated tolerable daily intake), but rather on a desire to keep levels as low as technologically feasible (basis for regulations in some countries), or 'free' of aflatoxins by not allowing residues above the analytical detection limit (basis for regulations in some other countries).

The allowable levels of aflatoxins in animal feeds and human foods vary with governmental jurisdictions (Coppock & Christian, 2007). The first legislative act was undertaken in 1965 by the Food and Drug Administration (FDA) which proposed a tolerance level of 0.03 ppb of total aflatoxins (Bl + Gl + B2 + G2). With increasing awareness of aflatoxins as potent toxic substances, the proposed level was lowered to 0.02 ppb in 1969. In 1973, the European Economic Community (EEC) established legislation on maximum permitted levels of AFBl in different types of feedstuffs. The legislation has been frequently amended since then (Ismail, 1997). Because of the

following reasons, it seems that monitoring and preventive program are the most effective strategies to decrease the risk of exposure to both human and animals: evaluation of human exposure levels and health risk based on animal toxicological research, assessing dietary intake and decontamination and removal of mycotoxins from human and animal diets.

Currently the limits of AFM1 in milk are highly variable, depending on the degree of development and economic status of the countries. European Communities and Codex Alimentarius have fixed the limit to a maximum of 0.05ppb (Mohammadi, 2011). The United States Food and Drug Administration have established action levels for aflatoxin at concentrations of 20 and 0.5 µg/kg for human food and milk, respectively (Chase *et al.,* 2013). In some countries, the maximum admissible level of AFM1 for children's food is 0.01ppb (FAO, 2004). Sixty countries now have regulations with respect to the presence of aflatoxin M1 in milk, with limits of 0.05–0.5 µg kg^{-1}; the European Union (EU) has a legal limit of 0.05 µg kg^{-1} (Driehuis, 2013). Kenya has adopted the Food and Agriculture Organization (FAO) and World Health Organization (WHO) limit of 10 ppb total aflatoxins and 5 ppb AFB1 in food.

2.9 Mitigation of aflatoxin (AFM1) occurrence in milk

Many methodologies have been developed to reduce AFM1 contamination with both direct and indirect approaches being extensively reviewed (Jard *et al.,* 2011). These include good agricultural practices in pre-harvest and post-harvest management of feed

crops (including storage) and physical, biological or chemical decontamination of feed and milk.

Biocontrol refers to the use of organisms to reduce the incidence of toxigenic strains of *Aspergillus* in susceptible crops and may be implemented to reduce AFB1 concentrations in feed of dairy animals during both crop development and post-harvest storage thus indirectly reducing AFM1 contamination of milk. Different organisms, including bacteria, yeasts, and nontoxigenic *Aspergillus* strains, have been tested as competitive biocontrol agents. To date, the most successful biocontrol method employs nontoxigenic strains of *A. flavus* and *A. parasiticus*, applied with a carrier/substrate, such as a small grain, in fields where they competitively exclude the toxigenic strains and preferentially infect the susceptible crop (Yin *et al.*, 2008). Non-aflatoxigenic native *A. flavus* has been effective in significantly reducing aflatoxin contamination in fields of maize, groundnuts, and cottonseed (Atehnkeng *et al.*, 2008; Pitt and Hocking, 2006). Biocontrol to counteract aflatoxin contamination during storage has been tested with some success with probiotic yeast and bacterial strains. *Saccharomyces cerevisiae* resulted to be one of the most effective microorganisms for binding AFB1 (Shetty & Jespersen, 2006).

The use of clay-based enterosorbents in the diet of dairy animals may reduce absorption of AFB1 in the animal body thus reducing carry-over in milk. Significant reductions of the concentration of AFM1 in milk were observed when clay enterosorbents were included in the diet of lactating dairy cattle and goats fed with feed contaminated with

AFB1 (Phillips *et al.,* 2008). Studies have shown that in dairy cows, activated carbon (AC) and hydrated sodium calcium aluminosilicate (HSCAS) mixed to AFB1 contaminated feed with an inclusion rate of 2%, reduced AFB1 carry-over as AFM1 in milk of 50% and 36%, respectively (Galvano *et al.,* 1996). A study comparing the effects of AC, esterified galactomannan, calcium bentonite, and three hydrated sodium calcium aluminosilicate (HSCAS) products showed reductions in milk AFM1 concentrations of 5.4%, 59%, 31%, 65%, 50%, and 61%, respectively (Diaz *et al.,* 2004). The inclusion of two commercial HSCAS products, Novasil Plus® and Solis®, or an esterified galactomannan product (MTB-100) at 0.5% to the diet of dairy cows reduced milk AFM1 concentration by 45%, 48%, and 4%, respectively (Kutz *et al.,* 2009).

More recently, the ability of saponite-rich bentonite to reduce AFM1 contamination in milk was investigated. The detoxification capacity of the bentonites used was efficient, bringing contamination below the European standard limits for AFM1 (50 ng/kg), with moderate alteration of the nutritional properties of the milk. Bentonite residues retained in milk (0.4%) were of no concern for human health (Carraro *et al.,* 2014).

Probiotics, such as Lacto Acid Bacteria and *Saccharomyces sp*., have been frequently employed as binding agents, due to their Generally Recognized as Safe (GRAS) status, high binding abilities, and wide distribution in nature. Commercial *Lactobacillus* and *Streptococcus* strains have been shown to reduce to varying degrees AFM1 concentration in phosphate buffered saline (PBS), milk, and yoghurt (Ayoub *et al.,* 2011;

El Khoury *et al.*, 2011; Sarimehmetoglu & Kuplulu, 2004). The ability of different Lacto Acid Bacteria (LAB) to remove AFM1 from processed milk, such as yoghurt, has also been demonstrated (Elsanhoty *et al.,* 2014). Maximum AFM1 binding capability (100%) has been reported with a combination of *S. cerevisiae* and a pool of three heat-killed Lacto Acid Bacteria (*Lactobacillus rhamnosus*, *Lactobacillus delbrueckii spp*. *bulgaricus* and *Bifidobacterium lactis*) (Corassin, *et al.,* 2013).

Several fungal strains, including a non-toxigenic *A. flavus*, *A. niger*, *Eurotium herbariorum*, and *Rhizopus sp*., have been found to biotransform AFB1 into less toxic metabolites. However, their potential use in the food industry may be limited by the long incubation time required for detoxification (more than 72 hours), incomplete degradation, non-adaptation to typical food fermentations, and culture pigmentation (Guan *et al.,* 2011).

An entirely innovative strategy to decrease risks associated with contamination of feeds by aflatoxins, and their carry-over in milk and edible tissues, could rely on vaccination. Systemic vaccination of dairy cows and heifers has recently proved to be effective in reducing AFB1 carry-over as AFM1 in milk (Giovati *et al.,* 2014; Polonelli *et al.,* 2011). Vaccination with a mycotoxoid vaccine formulated with protein-conjugates of Anaflatoxin B1 (An-AFB_1), induces antibodies (Abs) that specifically block initial absorption or bioactivation of AFs, toxicity, and/or excretion in milk or other products, by immuno-interception (neutralization).

The use of physical methods including sorting, sieving, steeping, density segregation of grains and nuts has significantly reduced the aflatoxin content of grains. Sorting can be done either manually or commercially by use of electronic sorting machines (Awuah *et al*., 2009; Dorner, 2008). Studies have shown a reduction of aflatoxin content by 40-80% following physical cleaning and separation procedures of contaminated and physically damaged kernels (Park, 2002). Promotion of rapid and effective drying methods of grains after harvesting has also proved effective (Bruns, 2003). Grains should be dried to a moisture content of 12-15%.

Currently, attention is focused on the development of an efficient and sensitive method for the routine assay of AFM1 in milk and milk products. Progress has indeed been made toward the development of sensor devices for the rapid and in field determination of AFM1 in milk without highly skilled personnel (Goryacheva *et al.,* 2009). Immunosensors detect a signal generated from antigen–antibody interaction and convert it into a measurable signal.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was carried out in Bomet County which is one of the leading milk producing counties in Kenya due to its favorable climatic conditions. The selection of the sampling sites was done with the assistance of the County Livestock Production Officer together with staff from Veterinary, Animal production and Agriculture departments. Seven milk collection centers were selected based on their milk holding capacities which corresponded to the number of farmers supplying their milk there. The farmers were informed of the study and its importance by the milk cooling plants managers prior to sampling. Approval to carry out the study was granted by the County Government of Bomet.

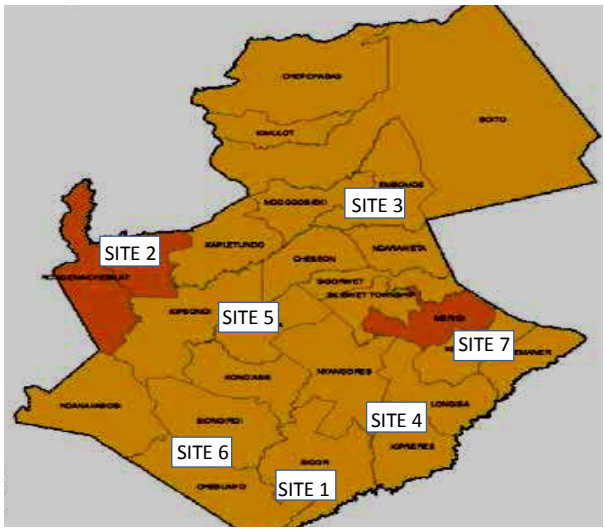


Figure 3-1: The study area (Bomet County), Kenya (*knbs.or.ke*)

3.2 Study Design

This was a cross sectional study where the milk collection centers/cooling plants were visited once to collect milk samples. Basic information on whether the farmers supplement grazing with other feeds, milking practices and milk handling was obtained by interviewer administered questionnaire. Processed milk and milk products were purchased randomly at the then market price from various milk kiosks/shops across the county.

3.3 Sample size determination

The minimum sample size was determined according to Fischer *et al.,* (1998).

$$n = \frac{z^2 \mathbf{P}(1-\mathbf{P})}{\boldsymbol{\delta}^2}$$

Where n = minimum sample size

z = Standard normal deviate that corresponds to 95% confidence interval (1.96)

P = Estimated prevalence

δ = is the level of significance (5%)

Since there is little information on the occurrence of AFM1 in milk and milk products in Kenyan rural set-ups a prevalence rate of 50% was used to calculate the sample size. Assuming this prevalence at 95% confidence interval the minimum estimated sample size was given as 384 using the formula given below.

$$\text{Therefore; } n = \frac{(1.96)^2 \times 0.50(1-0.50)}{(0.05)^2} = 384$$

The estimated total sample size of 384 (192 raw milk and 192 processed milk and milk products) could not be obtained as expected according to the sample size calculation with a prevalence of 50%. All raw milk samples (192) were collected, however, only thirty five processed milk and milk products were obtained since majority of the shops/kiosks did not have. Most of the residents consume fresh cows' milk and traditionally fermented milk products.

3.4 Sample collection, Transportation and Biosafety measures

One hundred and ninety two (192) raw cows' milk samples and thirty five (35) processed milk (fresh processed and Ultra Heat Treated) and milk products (fermented and yoghurt) were collected. All collected samples were given identification number for ease of tracing the results back to the particular farmers. The samples of fresh raw milk were collected aseptically from individual farmers as they brought their milk to the cooling plants/collection centers following the standard procedure. Briefly, the milk was shaken to mix and then transferred into sterile screw-capped sampling bottles (250ml capacities). The screw-capped sampling bottles were then securely capped and labeled with permanent markers. Processed milk and milk products were purchased from milk kiosks and shops. The samples were transported in cool boxes with ice bags to Mycology laboratory at the Centre for Microbiology Research, (KEMRI) where mycological and AFM1 investigations were done. Plastic sachets were cleaned with 70% ethanol and opened with sterile scissors whereas plastic bottles were aseptically sampled after mixing.

3.5 Mycological Investigation

Mycological investigation to determine the contaminating yeasts and molds in all the collected milk samples (192 raw and 35 processed milk and milk products) was carried out using standard mycological procedures (CLSI, 2012).

3.5.1 Primary Isolation

Sabouraud Dextrose agar supplemented with 1% chloramphenicol was used for primary isolation and cultivation of yeasts and molds. The media were prepared aseptically according to the manufacturer's instructions and dispensed in 25 ml amounts into 90 mm-diameter sterile petri dishes. This was done inside a clean bench laminar cabinet which was sterilized by wiping with 70% alcohol. Each petri dish was labeled with the identification number given to respective samples after solidification of agar. Briefly 100µl of the milk (raw, fresh processed and UHT) and milk products (fermented and yoghurt) were inoculated onto the center of the SDA plates and spread using sterile glass beads. Incubation was done at 30°C for 3-5 days and checked daily for any growth before sub-culturing positive ones.

3.5.2 Purification

Colonies with distinct morphological differences such as color, shape and size were picked using a wire loop sterilized by heating over a Bunsen burner and purified on Sabouraud dextrose agar plates supplemented with 1% chloramphenicol. The sub-cultures were incubated at 30°C for 2-5 days. Pure isolates were stocked at -20^{o}C on glycerol media before identification. The isolated species were identified on the basis of

the macromorphological properties of colonies and micromorphological properties of conidial and other structures, and according to the key described by Larone, (1995).

3.6 Identification of yeasts

For identification of yeasts, primary classification of colonies from the SDA agar plates was based on colony characteristics (pigmentation and shape).

3.6.1 Identification of yeasts on CHROMagar

Purified yeast colonies from Sabouraud agar were sub-cultured onto CHROM agar (CHROM agar Candida, Paris, France) for preliminary identification of yeasts and to detect mixed cultures as described by Sivakumar *et al*., (2008). CHROM agar contains a chromogenic, 5-bromo-6-chloro -3- indolyl phosphate P-toluidine (chromogenic substrates) and 5 bromo-4-chloro-3-indolyl/ N-acetyl-β-D-glucosaminide, which reacts with species specific enzymes to give yeasts colonies which are different in color (Ghelardi *et al.*, 2008). Preparation of media was done according to the manufacturer's instruction aseptically and then dispensed in 25 ml amounts into 90 mm-diameter Petri dishes. Purified yeast colonies on Sabouraud agar were inoculated onto CHROM agar using a sterile wire loop followed by incubation at 30°C for 48 hours.

3.6.2 Identification of yeasts on Cornmeal agar

Dalmau plate culture was set up for colonies that could not be identified using CHROM agar. It tests the ability of yeasts to produce pseudohyphae, true hyphae, arthrospores and chlamydospores (Peter & Joachim, 2006; Kurtman & Fell, 2000; WHO, 2009). Colonies from CHROM agar were transferred, inside a biosafety cabinet using a sterile

wire to cornmeal agar by making a parallel cut of approximately ½ inch into the agar and inoculating by holding the wire at an angle of 45°C and a clean sterile cover slip put onto the agar, covering a portion of the inoculation streaks. The incubation of the inoculated plates was done at 35°C for 3 to 4 days. The cultures were examined microscopically, through the cover slip to prevent contamination of the microscope objective with the agar and dislodging of the conidia. The preparation was examined ×400 magnification, for the presence of characteristic pseudohyphae, true hyphae, arthrospores and chlamydospores.

3.6.3 Identification of *Rhodotorula* species

Rhodotorula sp. was identified based on their colony color on primary isolation media (SDA) which was red-orange to orange due to the production of carotenoids (Larone, 2002).

3.6.4 Identification of *Cryptococcus* species

Suspected yeast colonies were sub-cultured onto Sabouraud Dextrose agar to purify cultures. The procedures were done according to the manufacturer's instructions using aseptic techniques. Indian ink test was then set up for presumptive identification. Briefly a drop of Indian ink was placed on a glass slide and mixed with suspected colonies. A cover slip was then added and the slide examined for the presence of encapsulated cells as indicated by clear zones surrounding the cells. Determination of the carbohydrates, which *Cryptococcus sp.* is able to use as the sole source of nutrients, was performed using the API 20C AUX (bioMérieux, France) system. Identification was then made by

comparing the generated numerical profile to the corresponding species in the Analytical Profile Index 20 C AUX chart.

3.6.5 Identification using Analytical Profile Index (API 20 C AUX)

Confirmation of the yeasts isolates was done by using rapid miniaturized system of carbohydrate assimilation profiles API 20 C AUX (bioMérieux, France) according to Kurtzman *et al.,* (2003). As recommended by the manufacturer, each isolate was subcultured prior to testing, to ensure viability and purity. Yeast inoculum suspensions were prepared from 18-24 hours cultures grown on Sabouraud dextrose agar plates at 35°C. The incubation box (tray and lid) was prepared by distributing about 5 ml of distilled water or demineralized water into the honeycombed wells of the tray to create a humid atmosphere. The strain reference was recorded on the elongated flap of the tray. The strips from their individual packaging were removed and placed in the incubation tray.

To prepare the inoculum, ampules of API Suspension Medium (2 ml) were opened aseptically and using a pipette, portions of yeast colonies were picked by successive touches. Suspensions with turbidity equal to 2 McFarland standards were prepared and 100 µl of it transferred to ampules of API C Medium transfer immediately. It was then gently homogenized with a pipette, avoiding the formation of bubbles.

Inoculation of the strip was done by filling the cupules with the suspension obtained in the ampule of API C Medium by placing the tip of the pipette against the side of the cupule to avoid formation of bubbles taking care not to overfill or underfill the cupules.

The lid was then placed on the tray after which the reactions were read visually after 24, 48 and 72 hours of incubation at 35°C by comparing the turbidity of the cupules with that of controls.

A numerical profile of a 7-digit number was deduced by adding the numbers corresponding to positive reactions within groups of 3 as given on the result sheet. Identification was then made by comparing the generated numerical profile to the corresponding species in the Analytical Profile Index 20 C AUX chart.

3.7 Identification of molds

Morphological features of molds were studied and the major macroscopic features such as colony diameter, colony color on agar and reverse and colony texture were used to aid identification. Each morphologically different mold colony from the SDA plates supplemented with chloramphenicol was picked up, transferred to SDA and incubated for 5 days at 30 °C.

3.7.1 Lactophenol cotton blue stain test

Lactophenol cotton blue stain was used for staining and microscopic identification of molds (Forbes *et al.,* 2002). Briefly a drop of lacto phenol cotton blue stain was placed in the center of a clean slide and a small (no more than 2-3 mm) portion of the colony near the margin was picked carefully using mycological needles. This was followed by teasing using the needles so that the filaments were well spread and clean sterile cover slips applied gently.

All the procedures were done in the level II biosafety cabinet. The LPCB mounts were examined at x400 magnification. The hyphal structure, spore type, shape and arrangement were noted and used in the identification of the isolates. Microscopic characteristics that helped in the identification process were conidia heads, stalks, color and length, vesicles shape, metula covering, conidia size, shape and roughness (Diba *et al.*, 2007).

3.8 Analysis of Aflatoxin M1 in milk samples

A total number of 185 samples (150 raw milk and 35 processed milk and milk products) were analyzed for aflatoxin M1. The less number of the analyzed samples (185) from the initial of 227 was due to the unavailability of finances to purchase enough AFM1 Elisa kit.

3.8.1 Sample Preparation for AFM1 Detection

To perform the tests, the samples were allowed to thaw at room temperature, and then centrifuged at 3500 rpm for 10 minutes. The upper cream layer was then removed by aspirating with a Pasteur pipette. Fifty microliters of the milk samples were used directly for AFM1 assay.

3.8.2 Aflatoxin M1 Detection

The concentration of AFM1 was determined by direct competitive ELISA, using a RIDASCREEN Aflatoxin M1 test kit of Art. No: R5812 (R-Biopharm, Darmstadt, Germany). Briefly, 50µl of standard solutions and prepared samples were added into microtiter wells using a sterile pipette tip for each standard or sample. Exactly 50µl of

enzyme conjugate (peroxidase) was then added to each well. Another 50µl of anti-aflatoxin M1 was added into each well and mixing done gently by shaking the plate manually followed by incubation for 10 minutes at room temperature. The liquid was then poured out of the wells and the microwell holder tapped upside down onto a clean filter towel to remove all remaining liquid from the wells. This was then followed by washing twice with 250µl washing buffer.

The wells were emptied to remove all the remaining liquid. After that, 100µl of substrate/chromogenic solution (tetramethylbenzidine) was added to the wells, mixed gently by shaking the plate and incubated for 5 minutes at room temperature in the dark. Finally, 100µl of the stop solution was added into the wells mixed gently by shaking the plate manually and immediately the absorbance reading was taken photometrically at 450 nm in an ELISA reader. The absorbance is inversely proportional to the aflatoxin concentration in the sample i.e., the lower the absorbance, the higher the aflatoxin concentration.

3.9 Data Management and Analysis

3.9.1 Data Entry and Cleaning

Using Microsoft Excel® 2007 template, raw data was entered, cleaned and coded for analysis.

3.9.2 Data Analysis

Data was analyzed using STATA version 13 SE. Categorical variables were summarized as frequencies and the corresponding percentages. Continuous variables were

summarized using the median and the corresponding lower and upper quartiles as well as the minimum and the maximum values. Overall and site specific analyses were done.

Gaussian assumptions for the continuous variables were assessed empirically using Shapiro-Wilk test and graphically using normal probability plots. Association between categorical variables was assessed using Pearson's Chi Square test. Fisher's exact test was performed whenever the Chi Square assumptions were violated. The relationship between the absorbance levels and the aflatoxin concentration was studied using a cubic regression model. The choice of the non-linear model was guided by the relationship between the observed values of the two variables. Plausibility of the cubic regression model was assessed by comparing this model to those of lower order polynomials. Comparison was done using Pearson's Chi Square test. Evidence of the presence of aflatoxin M1 in the milk was determined using WHO/FAO acceptable limits of $0.05\,\mu g/l$. Results were presented using tables and graphs.

3.10 Ethical Considerations

All protocols and procedures used in the study were reviewed and approved by KEMRI's Scientific Steering Committee (SSC) and Ethical Review Committee (ERC); SSC Protocol Number 2890 prior to the study. Consent from individual farmers was sought before sampling their milk by having them read and sign the consent form. Laboratory procedures were performed in accordance to Standard Operating Procedures and Kenya Medical Research Institute Biosafety guidelines available in Mycology laboratorory.

CHAPTER FOUR

RESULTS

4.1 Dairy farmers' practices across the study sites in Bomet County

An interviewer administered questionnaire was used to obtain basic information on farmers' milking practices, milk handling and whether the farmers supplement grazing with other feeds. As indicated in Table 4.1, majority of the farmers 191(99.5%) cleaned the cows' udder before milking. It was noted that most of them did not use detergents in cleaning of udder 4(2.1%) rather they used warm 178(93.7%) and cold 9(4.7%) water respectively. All the farmers cleaned their milking containers with 15(7.8%) and 177 (92.2%) using cold and warm water respectively. Moreover, 72.9% of the farmers used detergents in cleaning their milking containers.

A combination of nappier grass and grass in the fields (pasture) formed the major part of the cows' feeds according to 79(41.1%) of farmers. Other feeds given included grass in the field, and a combination of spoilt grains, nappier grass and grass in the field according to 50(26.0%) and 35(18.2%) of farmers respectively. Twenty eight (14.6%) of farmers fed their cows grass in the field (pasture) and spoilt grains. Majority of the farmers 146(76%) also gave commercial feeds to their cows purchased from the various agro vets in the County.

Table 4-1: Dairy farmers' practices across the study sites in Bomet County

Characteristic		Sites							Total
		Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	
Milking area	In the open	2 (10.0%)	2 (9.5%)	5 (15.6%)	9 (40.9%)	14 (40.0%)	9 (29.05)	7 (22.6%)	48 (25.0%)
	Roofed crush	15 (75.0%)	17 (81.0%)	27 (84.4%)	13 (59.1%)	19 (54.3%)	22 (71.0%)	24 (77.4%)	137 (71.4%)
Cleaning the udder		20 (100.0%)	21(100.0%)	32 (100.0%)	22 (100.0%)	35 (100.0%)	31 (100.0%)	30 (96.8%)	191 (99.5%)
Substance used for	Cold water	0 (0.0%)	1 (4.8%)	0 (0.0%)	1 (4.5%)	5 (14.7%)	0 (0.0%)	2 (6.5%)	9 (4.7%)
cleaning the udder	Warm water	19 (95.0%)	19 (90.5%)	32 (100.0%)	21 (95.5%)	29 (85.3%)	31 (100.0%)	27 (87.1%)	178 (93.2%)
	Use of detergents	1 (5.0%)	1 (4.8%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (6.5%)	4 (2.1%)
Substance used for	Cold water	2 (10.0%)	4 (19.0%)	0 (0.0%)	5 (22.7%)	1 (2.9%)	0 (0.0%)	3 (9.7%)	15 (7.8%)
cleaning containers	Warm water	18 (90.0%)	17 (81.0%)	32 (100.0%)	17 (77.3%)	34 (97.1%)	31 (100.0%)	28 (90.3%)	177 (92.2%)
Use of detergents		11 (55.0%)	12 (57.1%)	18 (56.2%)	18 (81.8%)	29 (82.9%)	28 (90.3%)	24 (77.4%)	140 (72.9%)
Cow's feeds	Grass in the field	10 (50.0%)	3 (14.3%)	9 (28.1%)	7 (31.8%)	6 (17.1%)	4 (17.1%)	11 (35.5%)	50 (26.0%)
	Napier grass & Grass in the field	5 (25.0%)	9 (42.9%)	20 (62.5%)	7 (31.8%)	12 (34.3%)	12 (38.7%)	14 (45.2%)	79 (41.1%)
	Spoilt grains & Grass in the field	2 (10.0%)	1 (4.8%)	1 (3.1%)	4 (18.2%)	10 (28.6%)	6 (19.4%)	4 (12.9%)	28 (14.6%)
	grains, Napier & Grass in field	3 (15.0%)	8 (38.1%)	2 (6.2%)	4 (18.2%)	7 (20.0%)	9 (29.0%)	2 (6.5%)	35 (18.2%)
Gives commercial feeds		13 (65.0%)	18 (85.7%)	23 (71.9%)	13 (59.1%)	22 (62.9%)	27 (87.1%)	30 (96.8%)	146 (76.0%)

4.2 Mycobiota in milk and milk products from Bomet County, Kenya

The occurrence of yeasts and molds in milk and its products from Bomet County were either as single or multiple infestations in all the samples analyzed. There were three hundred and sixty five fungal isolates of which 27 (7.4%) were molds and 338 (92.6%) were yeasts (Figure 4.1). Site 3, Site 4, Site 5, Site 6 and Site 7 had a higher proportion of yeasts compared to molds. All the processed milk samples showed no growth on primary isolation media.

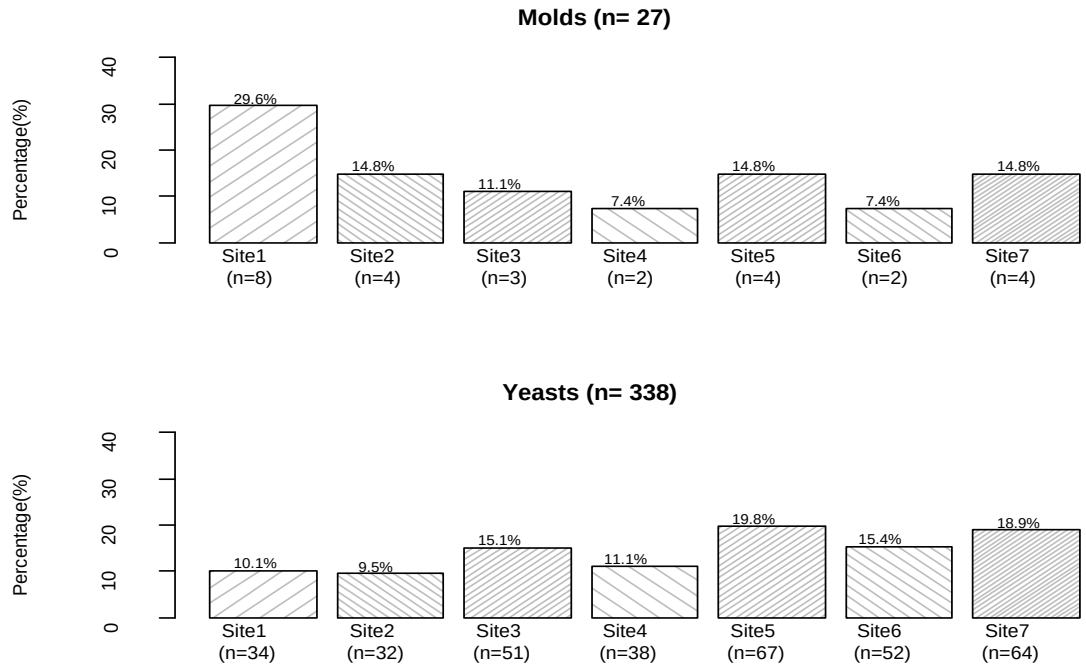


Figure 4-1: Percentage distribution of molds and yeasts isolated from milk samples obtained from farmers across the milk collection sites in Bomet County.

4.3 Isolated fungal species in raw milk samples obtained from farmers in Bomet County.

The isolated yeasts and molds in milk were identified based on culture, morphological and microscopic characteristics as summarized in Appendix 1 and Appendix 2 respectively. The isolates were classified and grouped into 7 genera. Candida was the predominant genera isolated at 57.8% (211/365). Others were Rhodotorula 27.1% (99/365), Saccharomyces 6.6% (24/365), Geotrichum 4.1% (15/365) Aspergillus 3% (11/365), Cryptococcus 1.1% (4/365) and Fusarium 0.3% (1/365) (Figure 4.2). Plates 4.1 shows the fungal contamination obtained in some of the milk sampled.

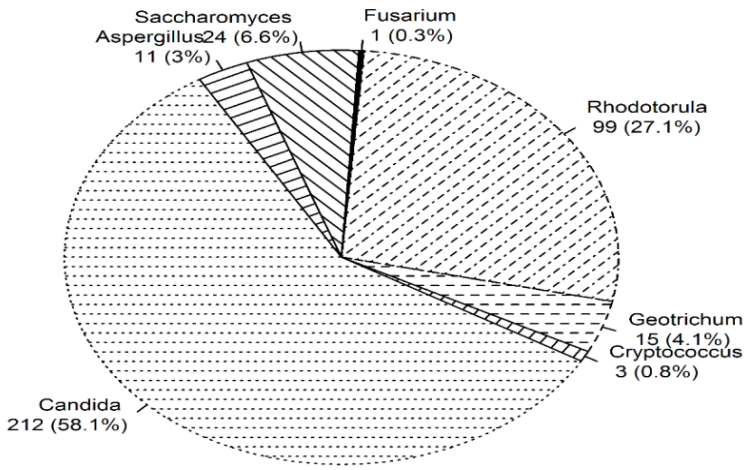
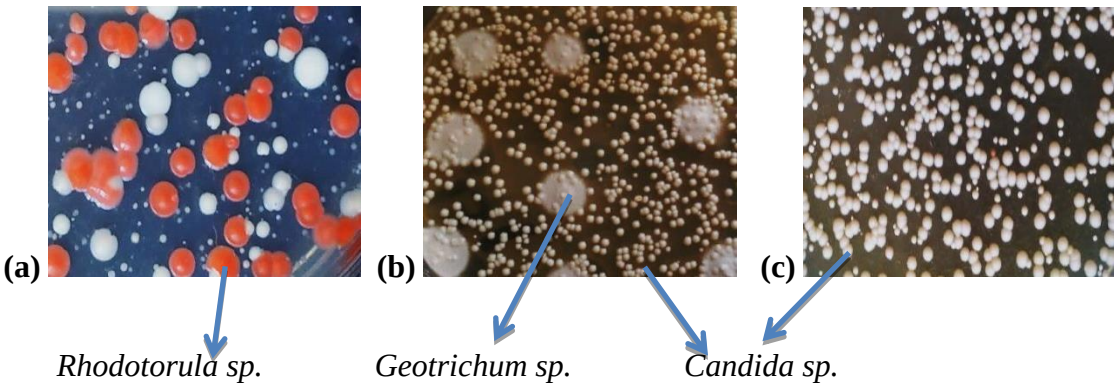


Figure 4-2: Percentage distribution of fungal genera isolated from raw milk samples obtained from farmers at milk collection/cooling sites in Bomet County.



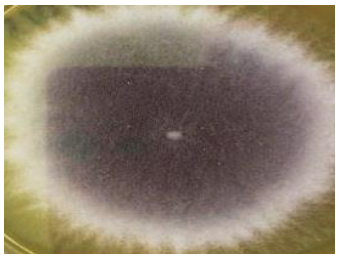
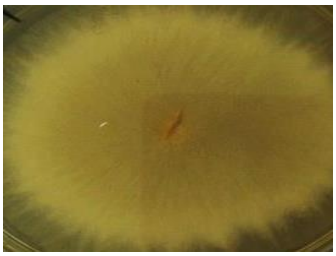
Plates 4-1 a, b and c: Phenotypic features of isolated yeasts and yeast like fungi on SDA at x400 magnification

4.3.1 Filamentous fungi identified from raw milk samples obtained from farmers in Bomet County

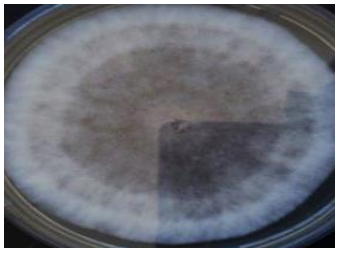
Out of a total of 365 fungi isolated, 27 were identified macroscopically and microscopically as molds. Majority of which were *Geotrichum sp.*, 15/27 (55.6%). Others were *Aspergillus fumigatus* 7/27 (25.9%), *A. niger* 2/27 (7.4%), *A. versicolor* 2/27 (7.4%) and *Fusarium sp*. 1/27 (3.7%) (Table 4.2).

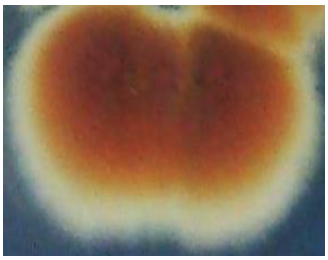
Table 4-2: Different types of filamentous fungi isolated from raw milk samples obtained from farmers in Bomet County

Types of filamentous fungi	Frequency (%)
Geotrichum sp.	15 (55.6%)
Aspergillus fumigatus	7 (25.9%)
Aspergillus niger	2 (7.4%)
Aspergillus versicolor	2 (7.4%)
Fusarium sp.	1 (3.7%)
TOTAL	**27 (100%)**

a (ii)  **a (ii)**  *Aspergillus niger*

b (i)  **b (ii)**  *Fusarium sp.*

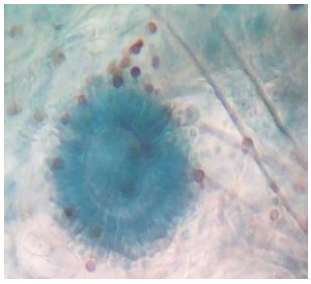
c (i)  **c (ii)**  *Geotrichum sp.*

d (i)  **d (ii)**  *Aspergillus versicolor*

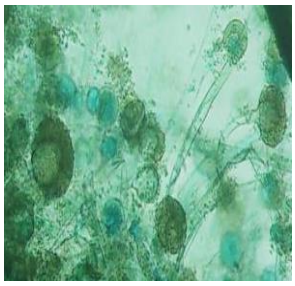
Plates 4-2 (a-d): Colonial (macroscopic) morphologies of isolated molds from raw milk samples growing on Sabouraud Dextrose Agar

Plate 4.2 a: *Aspergillus niger* with initial growth as white felt-like mat of mycelia, becoming black later on giving "salt and pepper appearance" as a result of darkly pigmented conidia borne in large numbers **a (i)** and reverse turning pale yellow in color **a (ii)**. **Plate 4.2 b:** *Fusarium sp.* characterized by slow-growing, smooth, velvety- like, pink with white margin colony **b (i)**. Reverse is pale in color **b (ii)**. **Plate 4.2 c:** *Geotrichum sp.* is characterized by velvety or suede-

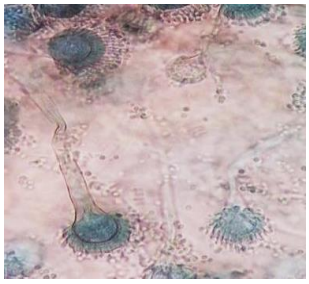
like, flat, white to cream colored with low aerial mycelium **c (i)** exhibiting rapid growth with no reverse pigmentation **c (ii)**. **Plate 4.2 d:** *Aspergillus versicolor* characterized by pale greyish green color **d (i)**. Reverse is reddish-brown in color **d (ii)**.

(a)  x400

Aspergillus versicolor

(b)  x400

Aspergillus niger

(c)  x400

Aspergillus fumigatus

Plates 4-3 (a-c): Microscopic morphologies of lacto phenol cotton blue preparations of some isolated molds from milk at magnification of x400.

Plate 4.3a shows *Aspergillus versicolor* with long hyphae, biseriate, conidial heads and spherical conidia. *Aspergillus niger* is characterized by large dark brown, biseriate conidial heads, smooth-walled conidiophores and dark-brown conidia (**Plate 4.3b**). **Plate 4.3c** shows *Aspergillus fumigatus* with uniseriate conidial heads short smooth-walled conidiophores, conical shaped terminal vesicle and round conidia.

4.3.2 Filamentous fungi isolated from raw milk samples across the study sites in Bomet County.

The highest proportion of molds was observed in Site 1 with a percentage of 29.6% (8/27). Site 4 and Site 6 had the least number of molds both at 7.4% (2/27). Others were Site 2, Site 5 and Site 7 which had 14.8% (4/27) molds respectively. Site 3 had 11.1% (3/27) molds (Table 4.3). The genus *Geotrichum* was isolated in all the sites except Site 4. *Fusarium* was the least found genus with only one isolate from Site 2.

Table 4-3: Types of filamentous fungi isolated from raw milk samples obtained from farmers across the study sites in Bomet County.

MOLDS	SITE1	SITE2	SITE3	SITE4	SITE5	SITE6	SITE7
A. fumigatus	2(25%)	1(25%)	1(33.3%)	2(100%)	0(0%)	1(50%)	0(0%)
A. niger	1(12.5%)	0(0%)	0(0%)	0(0%)	1(25%)	0(0%)	0(0%)
A. versicolor	1(12.5%)	1(25%)	0(0%)	0(0%)	0(0 %)	0(0%)	0(0%)
Fusarium sp.	0(0%)	1(25%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
Geotrichum sp.	4(50%)	1(25%)	2(66.7%)	0(0%)	3(75%)	1(50%)	4(100%)
TOTAL NO. PER SITE	**8(29.6%)**	**4(14.8%)**	**3(11.1%)**	**2(7.4%)**	**4(14.8%)**	**2(7.4%)**	**4(14.8%)**

*A: *Aspergillus*

4.3.3 Types of yeasts isolated and identified from raw milk samples obtained from farmers across milk collection sites in Bomet County.

Majority of the yeasts isolated were from the genus *Candida 195/338* (57.8%) and it was the most diverse, with 211 isolates and 12 species. *Candida glabrata* was the most frequently isolated species at 47/338 (13.9%) whereas *C. humicola* 2/338 (0.6%) was the least isolated. *Rhodotorula sp.*, *Saccharomyces sp.* and *Cryptococcus sp.* made up 99/338 (27.1%), 24/338 (8.6%) and 4/338 (1.1%) of the yeast isolates, respectively (Table 4.4).

Table 4-4: Different types of yeasts isolated from raw milk samples obtained from farmers across milk collection sites in Bomet County.

Types of yeasts	Frequency (%)
Rhodotorula sp.	99 (29.3%)
Candida glabrata	47 (13.9%)
Candida parapsilosis	30 (8.8%)
Candida albicans	28 (8.3%)
Candida tropicalis	28 (8.3%)
Saccharomyces sp.	24 (7.1%)
Candida kefyr	20 (5.9%)
Candida lusitinae	18 (5.3%)
Candida krusei	13 (3.9%)
Candida famata	8 (2.4%)
Candida guilliermondii	7 (2.1%)
Candida lipolytica	7 (2.1%)
Cryptococcus albidus	4 (1.2%)
Candida rugosa	3 (0.9%)
Candida humicola	2 (0.6%)
TOTAL	**338 (100%)**

Site 5 had the highest number of yeasts i.e. 67/338 (19.8%) while Site 2 recorded the lowest number at 32/338 (9.5%). Others were Sites 7, 6, 3, 4 and 1 with 64/338 (18.9%), 52/338 (15.4%), 51/338 (15.1%), 38/338 (11.2%) and 34/338 (10.1%) yeasts respectively (Table 4.5).

Table 4-5: Distribution of yeasts from raw milk samples obtained from farmers across milk collection sites in Bomet County.

YEASTS	SITE1	SITE2	SITE3	SITE4	SITE5	SITE6	SITE7
C. albicans	4(11.8%)	3(9.4%)	5(9.8%)	5(13.2%)	0(0%)	3(5.8%)	8(12.5%)
C. famata	1(2.9%)	1(3.1%)	1(2.0%)	1(2.6%)	0(0%)	3(5.8%)	1(1.6%)
C. glabrata	3(8.8%)	3(9.4%)	10(19.6%)	4(10.5%)	12(17.9%)	6(11.5%)	9(14.1%)
C. guilliermondii	1(2.9%)	0(0%)	1(2.0%)	1(2.6%)	0(0%)	0(0%)	4(6.3%)
C. humicola	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	2(3.9%)	0(0%)
C. keyfr	1(2.9%)	3(9.4%)	5(9.8%)	2(5.3%)	3(4.5%)	3(5.8%)	3(4.7%)
C. krusei	0(0%)	1(3.1%)	0(0%)	1(2.6%)	5(7.5%)	2(3.9%)	4(6.3%)
C. lipolytica	0(0%)	2(6.3%)	0(0%)	1(2.6%)	2(3.0%)	1(1.9%)	1(1.6%)
C. lusitinae	3(8.8%)	3(9.4 %)	1(2.0%)	2(5.3%)	4(6.0%)	3(5.8%)	2(3.1%)
C. parapsilosis	1(2.9%)	1(3.1%)	7(13.7%)	6(15.8%)	6(9.0%)	3(5.8%)	6(9.4%)
C. rugose	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	3(4.7%)
C. tropicalis	3(8.8%)	2(6.35)	5(9.8%)	3(7.9%)	2(3.0%)	9(17.3%)	4(6.3%)
Rhodotorula sp.	14(41.2%)	11(34.4%)	11(21.6%)	10(26.3%)	23(34.3%)	15(28.9%)	15(23.4%)
Saccharomyces sp.	2(5.9%)	2(6.3%)	5(9.8%)	2(5.3)	8(11.9%)	2(3.9%)	3(4.7%)
Cr. albidus	1(2.9%)	0(0%)	0(0%)	0(0%)	2(3.0%)	0(0%)	1(1.6%)

*C: *Candida*, Cr: *Cryptococcus*

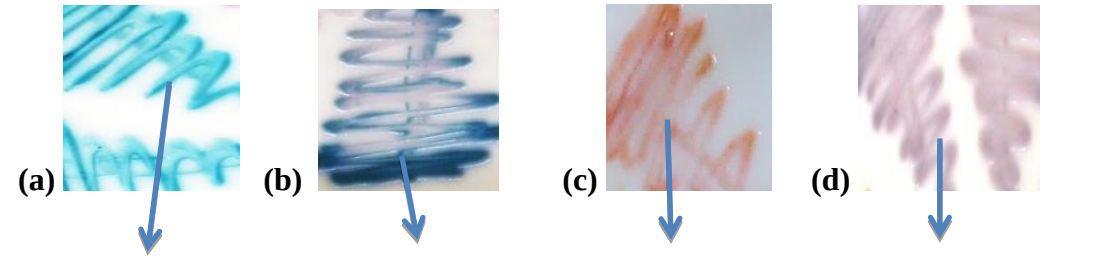
4.3.4 Identification of *Rhodotorula sp.*

Rhodotorula sp. colonies on primary isolation media (SDA) appeared red-orange to orange due to the production of carotenoids. It showed large round blastospores in cornmeal agar; pseudohyphae and hyphae were absent.

Plate 4-4: Smooth, round, glistening soft and mucoid red-orange colonies of *Rhodotorula* species.

4.3.5 Identification of yeasts on Chromagar

The CHROM agar supported the growth of most yeast isolates and its opaque to white background allows good discrimination among colonies of different species with almost similar hues. A wide variety of colony colors were seen some which were species specific. *Candida albicans* appeared greenish blue, *C. tropicalis* dark blue, *Rhodotorula sp.* red-orange, whereas other yeast species had various shades of purple and pink (Plates 4.5).

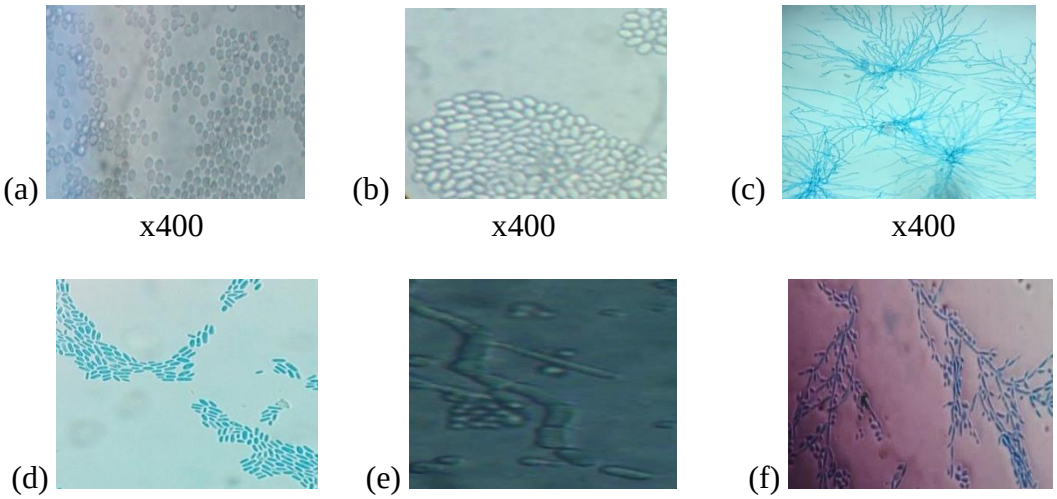


Candida albicans (Greenish blue) | *Candida tropicalis* (Deep blue) | *Rhodotorula sp.* (Brick red to orange) | Other yeasts (Shades of purple)

Plates 4-5 (a-d): Isolated yeast species on CHROMagar Candida media

4.3.6 Identification of yeasts on cornmeal agar

All the yeast species which were not identified using CHROM agar were plated on cornmeal agar to study the formation of pseudohyphae, true hyphae, arthrospores and chlamydospores.



Plates 4-6: Microscopic appearance of fungal isolates obtained from raw milk samples on cornmeal agar

Plates 4.6: Plate a: Microscopic appearance of *Rhodotorula species* on cornmeal agar showing large round to oval blastospores; **Plate b:** *Saccharomyces sp.* showing oval blastospores; **Plate c:** *Candida parapsilosis* characterized by curved and large hyphae; **Plate d:** *Candida kefyr* showing elongate blastospores in chains; **Plate e:** *Geothricum sp.* characterized by rectangular arthrospores breaking off and **Plate f:** *Candida tropicalis* characterized by long, wavy pseudohyphae with numerous ovoid blastoconidia.

4.3.7 Confirmation of yeast species using API 20C AUX

Analytical Profile Index 20C AUX which analyses each species based on carbohydrates assimilation profile was able to confirm yeast species some which could not be identified on cornmeal agar. The identification of each species was possible by referring to the manufacturers analytical profile index provided by Bio Merieux France. The yeasts were confirmed as *C. lusitinae, C. humicola, Cr. albidus, C. rugosa, C. famata* and *Saccharomyces cerevisiae* (Table 4.6).

Table 4-6: Yeast species identified using API 20 C AUX

API 20Caux ID Profile	Yeast Cells
6776375	*Candida lusitinae*
6576777	*Candida famata*
6062004	*Candida rugose*
6777777	*Candida humicola*
2040000	*Saccharomyces cerevisiae*
2724273	*Cryptococcus albidus*

ID- Identification Number

4.4 Aflatoxins (AFM 1) in milk samples obtained from Bomet County

The occurrence of AFM1 in the milk samples is summarized in Table 4.7. The ELISA reader gave absorbance readings from which percentage absorbance was calculated. For standard solutions, the percentage absorbance was plotted against aflatoxin concentration to get the calibration curve (Appendix 3). From the calibration curve, aflatoxin concentrations for samples were calculated. Out of 185 samples analyzed 81/185 (43.8%) were positive for aflatoxin M1 at concentration beyond the threshold limit mainly contributed by the raw milk 78/150 (52.0%) compared to processed milk 3/35 (8.6%). Seventy two (48%) raw milk and 3(8.6%) processed milk samples were contaminated at concentrations below the threshold limit of $0.05\,\mu g/l$. Twenty nine (82.9%) processed milk samples were not contaminated.

Table 4-7: Occurrence of aflatoxin M1 in processed and raw milk samples obtained from Bomet County

Contamination	Raw	Processed	p-value
Concentration > 0.05 μg/l	78 (52%)	3 (8.6%)	
Concentration > 0 < 0.05 μg/l	72 (48%)	3 (8.6%)	<0.0001
Concentration = 0.00 μg/l	0 (0%)	29 (82.9%)	

Processed milk did not show any evidence of contamination, median 0.00 (IQR: 0.00, 0.00) $\mu g/l$ while the raw milk showed evidence of Aflatoxin M1 contamination, median 0.09 (IQR: 0.00, 0.50) $\mu g/l$ (Table 4.8). The overall median for all the milk samples was 0.02 (IQR: 0.00, 0.40) $\mu g/l$.

Table 4-8: Aflatoxin M1 levels (μg/l) in processed and raw milk samples obtained from Bomet County

Category of Milk	No. of Samples	Median	Lower Quartile	Upper Quartile	Minimum	Maximum
Raw	150	0.09	0.00	0.50	0.00	2.93
Processed	35	0.00	0.00	0.00	0.00	0.69

4.4.1 Aflatoxin M1 in raw milk samples obtained from farmers across study sites in Bomet County

The occurrence of AFM1 in Site 3 was high, 73.1% (19/26), compared to the other sites. Raw milk had the highest levels of contamination. Site 4 had the lowest prevalence, 37.5% (6/16) compared to the other sites although the test for differences was not statistically significant, P = 0.217 (Table 4.9).

Table 4-9: The occurrence of aflatoxins (AFM1) in raw milk samples obtained from farmers across study sites in Bomet County

	Contaminated (Concentration > 0 < 0.05 μg/l)		
SITES	**YES**	**NO**	**p-value**
SITE 1	7 (50.0%)	7 (50.0%)	
SITE 2	6 (40.0%)	9 (60.0%)	
SITE 3	19 (73.1%)	7 (26.9%)	
SITE 4	6 (37.5%)	10 (62.5%)	0.217
SITE 5	14 (48.3%)	15 (51.7%)	
SITE 6	11 (44.0%)	14 (56.0%)	
SITE 7	15 (60.0%)	10 (40.0%)	

Table 4-10: Aflatoxin M1 levels (μg/l) in raw milk obtained from farmers across study sites in Bomet County

Sites	**No. of Samples**	**Median**	**Lower Quartile**	**Upper Quartile**	**Minimum**	**Maximum**
SITE 1	14	0.14	0.00	0.88	0.00	1.16
SITE 2	15	0.02	0.00	0.29	0.00	0.93
SITE 3	26	0.54	0.03	0.86	0.00	2.93
SITE 4	16	0.01	0.00	0.15	0.00	0.44
SITE 5	29	0.04	0.00	0.44	0.00	0.93
SITE 6	25	0.00	0.00	0.48	0.00	0.80
SITE 7	25	0.10	0.00	0.22	0.00	0.81
Total	150					

4.4.2 Aflatoxin M1 in processed milk and milk products obtained from Bomet County

The occurrence of AFM1 in the processed milk was low 3/32 (8.6%) and was contributed mainly by fermented milk 1(16.7%) and yoghurt 2(25.0%) (Table 4.11). Both fresh processed milk and Ultra Heat Treated milk had no contamination. There was no statistically significant difference in the level of contamination between fermented (processed) milk and yoghurt.

Table 4-11: Occurrence of aflatoxins (AFM1) in processed milk products obtained from Bomet County

Contamination	Fermented	Yoghurt	p-value
Concentration > 0.05 μg/l	1(16.7%)	2(25.0%)	
Concentration < 0.05 μg/l	2(33.3%)	1(12.5%)	1.000[f]
Concentration = 0.00 μg/l	3(50.0%)	5(62.5%)	

[f] Fisher's exact test was done due to violation of the Chi Square assumptions

There was no evidence of contamination in fresh processed milk and Ultra Heat Treated milk. However, fermented milk as well as the yoghurt showed evidence of contamination with their maximum values above $0.05\,\mu g/l$ (Table 4.12).

Table 4-12: Aflatoxin M1 levels (μ g/l) in processed milk and milk products collected from various milk shops/kiosks in Bomet County

Processed Milk and Milk products	No. of Samples	Median	Lower Quartile	Upper Quartile	Minimum	Maximum
Fermented Milk	6	0.00	0.00	0.00	0.00	0.24
Fresh Milk	15	0.00	0.00	0.00	0.00	0.00
UHT	6	0.00	0.00	0.00	0.00	0.00
Yoghurt	8	0.00	0.00	0.28	0.00	0.69
Total	35	0.00	0.00	0.00	0.00	0.69

CHAPTER FIVE
DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

The results of the present study showed that out of the total number of the raw milk samples examined, the occurrence of yeasts and molds were 92.6% (n=338) and 7.4% (n=27), respectively. The presence of yeasts and yeast-like fungi may trigger alterations in the milk and dairy products due to the release of extracellular enzymes such as lipases and proteinases (Chen *et al.,* 2003), which affect the quality and organoleptic characteristics influencing the shelf-life of the products. Other researchers have reported a higher or similar distribution of fungi in raw milk. In a previous study, molds and yeasts were detected in 63.3% and 95.0% of milk samples in Slovenia respectively (Torkar & Vengust, 2008). According to a study carried out in Serbia, it was reported that 57–100% of milk samples were contaminated with fungi (including both yeasts and molds with a higher percentage of yeasts isolated as compared to molds (Pesic-Mikulec *et al.,* 2005).

The high numbers of yeasts suggests that they are able to multiply in the milk and may result in spoilage or, conversely, in enhancement of the flavor of the fermented milk (Gadaga *et al.,* 2000). The main defects caused by yeasts are fruity, bitter or yeasty off flavors, gas production, discoloration changes and texture. Continued lactose fermentation could lead to increased acidity, gassiness and fruity flavors, while continued hydrolysis of protein and fat could contribute to bitter and rancid flavors as well as a softening of product texture (Soloiman *et al.,* 2011).

Processed milk and milk products had neither growth of yeasts or molds. This indicates that hygienic measures during production and handling in the dairy factories were up to standard. It also shows that the milk treatment processes are effective in destroying contaminating molds and yeasts. Microbial contamination of pasteurized milk can occur from different sources, inefficient pasteurization, contamination from the environments, and poor packaging, unsatisfactory sanitation and unsuitable storage temperature or a combination of these (Fulya, 2011).

According to this study, majority of the yeasts isolated from raw milk samples were from the genus *Candida* followed by *Rhodotorula, Saccharomyces* and *Cryptococcus.* Yeasts of the genus *Candida, Geotrichum*, *Saccharomyces, Rhodotorula, Cryptococcus* and *Trichosporon* were isolated from milk in a previous study (Bozena *et al.,* 2012). More recent studies have shown that common yeast genera/species isolated from milk include *Candida spp., Kluvermocyces spp. Debaryomyces hansenii Rhodotorula sp.* and *Cryptococcus sp.* (Callon *et al.,* 2007; Mallet *et al.,* 2012) most of which were also isolated in this study. The variability in the number of yeasts isolated across the study sites could be due to varying practices of hygienic standards with regard to milking and storage of milk by farmers (Table 4.1).

In the genus *Candida, C. glabrata* and *C. humicola* was isolated the most and the least at 13.9% and 0.6% respectively. The presence of *Candida* species in milk may be due to contamination by humans or contaminated environments since *Candida* are commensal

microorganisms on the mucus membranes of both humans and animals and are also frequently found in the environment (Wrobel *et al.,* 2008). *Candida species* comprising of *C. krusei, C. albicans, C. tropicalis, C. glabrata* and *C. lusitaniae, C. holmii, C. lambica* have been identified in the crude milk from the cow's teats (Ludmilla *et al.,* 2011). This is a clear indication that fresh raw milk can be contaminated as it is being retrieved from the cow's udder since these organisms are among the normal flora of the udder (Kabede *et al.,* 2007).

A previous study found species of non-*albicans Candida* isolates from milk among them *C. krusei, C. tropicalis* and *C. guilliermondii* (Santos & Marin, 2005). This is in agreement with the present study where non-*albicans Candida* species were also prevalent. Although *C. albicans* remains the most frequent cause of candidemia in both immunocompromised and immunocompetent patients, a number of reports have documented infections caused by *C. tropicalis, C. glabrata, C. parapsilosis, C. krusei* and *C. lusitaniae* (Miceli *et al.,* 2011; Rodrigues *et al.,* 2010). *Candida albicans* causes "thrush" of the oral cavity and genital tract, especially in immunocompromised people such as those with HIV/AIDS as well as the aged people and young children (Satana *et al.,* 2010). When found in high concentration in food and the host gastrointestinal tract presents appropriate conditions for opportunistic colonization, *Candida* species can propagate secreting toxic metabolites causing a wide variety of symptoms (Colombo *et al.,* 2006). Most pastoralist communities use raw unprocessed milk which could be a risk factor for infection especially in immunocompromised individuals.

Rhodotorula genus represented 29.3% of the yeasts with 99 isolates and was the most frequent basidiomycetic yeasts isolated. *Rhodotorula* species are opportunistic red yeasts that are widespread in nature and can be isolated from a variety of sources including air, soil, seawater, plants, milk and their products and the household environment (Kutty & Phillip, 2008; Vishniac & Takashima, 2010). *Rhodotorula* species have been recognized as emerging yeast pathogens in humans in the last two decades due to immunosuppression and invasive medical device technology (Chung *et al.,* 2002; Krzysciak & Macura, 2010). A previous study on fungal contamination of food distributed in hospitals to immunocompromised patients revealed that yeasts including *Rhodotorula* species were present (Tomsíková *et al.,* 2002). *Rhodotorula sp.* have been isolated from stool samples, indicating that these yeasts can survive in the extreme conditions of the gastrointestinal tract, however, it is still uncertain whether *Rhodotorula* species is capable of passing from the gastrointestinal tract into the bloodstream (Silva *et al.,* 2004).

Saccharomyces spp. is known majorly as a fermentative organism (yeast) (Belewu *et al.,* 2000; Uzeh *et al.,* 2006). *Saccharomyces cerevisiae* has been known to be of beneficial role in cheeses and fermented milk for example kefir and koumiss. It has been associated with the production of aroma compounds and stimulation of lactic acid bacteria, improvement of nutritional value and inhibition of undesired microorganisms (Jespersen, 2003). In other research, *S. cerevisiae* has been isolated from raw milk but in low numbers (van den Tempel & Jakobsen, 1998). In this study, 7.1% of *Saccharomyces sp.* was isolated. *Saccharomyces cerevisiae* has been considered as a well-established cause

of nosocomial acquired yeast infection (Lhern *et al.,* 2002). The most consistent risk factor for *S. cerevisiae* fungemia is the use of probiotics. Studies have shown that since the mid-1900s, roughly 60-100 cases of *S. cerevisiae* fungemia from cultured yogurts/probiotics have been identified (Cassone *et al.,* 2003; Enache-Angoulvant & Hennequin, 2005; Riquelme *et al.,* 2003). *Saccharomyces cerevisiae* can cause a wide variety of clinical syndromes, like liver abscess and esophagitis (Konecny *et al.,* 1999). Although the vast majority of reported cases occur in patients who are critically ill with significant comorbidities, *S. cerevisiae* should be considered a potentially dangerous micro-organism as there are cases causing fungemia in immunocompetent patients as well (Cassone *et al.,* 2003).

The least isolated species was *Cryptococcus sp.* at 1.2% with four isolates. The genus *Cryptococcus* has been recognized in fruit juice, milk, soil and pigeon droppings for more than 125 years (Springer *et al.,* 2010). *Cryptococcus albidus* is a common transient on human skin and it has also been reported as a rare cause of pulmonary, CNS and vaginal infection. It has been isolated from the dairy environment at limited numbers (Welthagen & Viljoen, 1998). There have however, been no outbreaks attributable to environmental sources and no reports of animal-to-human transmission. Human-to-human transmission is rare.

Filamentous fungi, unlike yeasts, are normally regarded as spoilage organisms in milk. They are typically present at lower levels than yeasts (Arora *et al*., 1991). Different types of molds produce toxic substances that were designated as mycotoxins. Some are

mutagenic and carcinogenic, some display specific organ toxicity and some are toxic by other mechanism (Deak, 2008). Out of the filamentous fungi isolated, the genera *Geotrichum* was the most abundant at 55.6% followed by *Aspergillus* (40.7%) and *Fusarium* at 3.7%. According to a previous study, the genus *Geotrichum* (51.5%) dominated over *Aspergillus* (33.8%) (Torkar &Vengušt, 2008). In another study, the most commonly isolated mold genera was the *Geotrichum* (76.5%), followed by *Fusarium* (45.3%) and *Aspergillus* (31.2%) (Jodral *et al.*, 1993). The species of the genus *Aspergillus, Penicillium* and *Fusarium* were isolated in raw milk (Pešić Mikulec *et al.*, 2005). *Aspergillus spp., Alternaria spp., Fusarium spp., Neurospora spp.* are the most common fungal species found in milk products (Saadia, 2010).

Geotrichum is a genus of fungi found worldwide in soil, water, air, and sewage, as well as in plants, cereals, and dairy products; it is also commonly found in normal human flora and is isolated from sputum and feces (William *et al.*, 2006). *Geotrichum sp.* is an extremely common fungus and is the causative agent of geotrichosis (Chagas-Neto *et al.*, 2008; Mahendra *et al.*, 2013). Pulmonary involvement is the most frequently reported form of the disease, but bronchial, oral, vaginal, cutaneous and alimentary infections have also been reported (Etienne *et al.*, 2008; Huamin *et al.*, 2013). Exogenous geotrichosis may arise from contact with contaminated soil, fruits or dairy products (Boutrou & Gueguen, 2005). Geotrichosis affects mainly the patients who are immunocompromised due to some underlying disease such as neoplasms, diabetes mellitus, leucosis, renal transplant and HIV.

In the genus *Aspergillus, A. fumigatus* (25.9%), *A. niger* (7.4%) and *A. versicolor* (7.4%) were isolated. The presence of *Aspergillus sp.* in the raw milk samples could possibly be attributed to contaminated soiled udders and teats, air, and contaminated forage provided to the cows during milking (Ghiasian *et al.,* 2011). Previous investigation recorded *Aspergillus sp.* which included *A. candidus, A. flavus, A. fumigatus, A. niger* and *A. terreus* among others in unprocessed or fresh bovine milk (Perween *et al.,* 2013). In a previous study, *Aspergillus* group was the most prevalent fungi in all examined cow's milk samples where, *A. niger* and *A. fumigatus* constituted 26 % of the total fungal isolates (Talaat *et al.,* 2014). Another study reported *Aspergillus niger* as the most common mold isolated in milk (Chhabra, 1998). *Aspergillus sp.* are usually regarded as spoilage microorganisms but may cause invasive disease, aflatoxicosis, and allergic reactions in humans especially those that are immunocompromised and/or on prolonged antibiotic therapy (Leila *et al.,* 2010; Quinn *et al.,* 2002).

Fusarium species was the least isolated among the molds at 3.7% (1/27). *Fusarium* is a large genus of filamentous fungi, part of a group often referred to as hypomycetes. Its presence in milk could be as a result of the environment where the cows are reared since *Fusarium sp.* is widely distributed and is associated with plants and are relatively abundant members of soil microbial community. *Fusarium sp.* can cause mycotoxicosis in humans following ingestion of food that has been colonized by the fungal organism. The main toxins produced by these *Fusarium* species are fumonisins and trichothecenes. Some studies have reported their presence in milk and cheeses with high water activity,

such as unripened and smear-ripened cheeses (Bachmann *et al*., 2005; Montagna *et al*., 2004).

The occurrence of aflatoxins in food is a serious global health problem, particularly in developing countries. Since milk and dairy products are a source of many nutrients, the presence of AFM1 is undesirable and strategies for reducing dietary exposure to AFM1 are important (Mohamadi & Alizadeh, 2010; Nilchian & Rahimi, 2012). For infants and young children, their exposure to contaminated milk and milk products puts them at high risk for ingestion of AFM1 toxin (Guo *et al.,* 2013; Sepehr *et al.,* 2012; Yapar *et al.,* 2008).

In this study, 43.8% samples were found to be contaminated with AFM1 above threshold levels (0.05ppb) contributed mainly by the raw milk which was 52.0% compared to processed milk with 8.6%. Raw milk samples showed evidence of Aflatoxin M1 contamination, median 0.09 with a minimum of 0.00 $\mu g/l$ and a maximum of 2.93 $\mu g/l$. The difference in contamination levels between processed and raw milk samples was statistically significant, ($p<0.0001$). The high prevalence rate and levels of AFM1 contamination in the raw milk samples indicate that lactating cows in Bomet besides freely grazing are fed with AFB1-contaminated feeds. However, the level of AFB1 in the feed of dairy cattle could not be measured in the present study. This could be spoilt maize grains left after harvest or poorly stored commercial feeds (Table 4.12). It has been reported that environmental temperature, humidity, and moisture content of

the feed as well as pH and mechanical damage to cereal grains resulted in higher AFB1 in animal feeds (Amer & Ibrahim, 2010).

Previous studies recorded lower incidences of AFM1 in milk samples in comparison to this that went above the EU maximum limits at 5%, 20% and 36.2% respectively (Al Zuheir & Abu Omar, 2012; Duarte *et al.,* 2013; Fallah, 2010). High incidence and concentrations of AFM1 in cow's milk have however, been reported elsewhere. It was reported that all of 240 raw cow milk samples collected from 80 milk tanks at a milk collecting center in the central region of Thailand were contaminated with AFM1 at an average concentration of 0.070 µg/l (Ruangwises & Ruangwises, 2010). More recent study in the Greater Addis Ababa have recorded a high incidence of AFM1 in milk than this where over 90% of the milk samples contained aflatoxin M1 levels that exceeded the European Union limit of 0.05 micrograms per litre (Gizachew *et al.,* 2016). Out of a total of 110 milk samples, only nine contained aflatoxin M1 levels below 0.05 micrograms per litre. All raw milk samples had detectable levels of aflatoxin M1.

The occurrence of AFM1 in raw milk samples per sites showed Site 3 as being with high rate of contamination with 73.1%, compared to the other sites. Site 4 had low prevalence, 37.5% compared to the others however the test for differences was not statistically significant, P=0.217. The median levels of aflatoxin M1 was high in sites 1, 3 and 7. However, these sites showed some evidence of samples that were not contaminated (minimum = 0.0). The sites that had median aflatoxin M1 levels below the WHO/FAO acceptable limits of 0.05 $\mu g/l$ were sites 2, 4, 5 and 6. However, looking at

the corresponding maximum aflatoxin levels there is evidence that some samples were highly contaminated based on the threshold value of $0.05\,\mu g/l$.

The variations in AFM1 levels among studies/sites could be associated to different reasons such as geographical region, country, season, feeding systems, farm management practices and analytical methods (Ayhan *et al.,* 2010). It could also be linked to the carryover rate of AFB1 as AFM1 in milk which varies widely among animals, days, and from one milking session to the next and it is greatly influenced by physiological factors such as diet and health status of animals. Previous studies have looked into the transfer of AFB1 in milk as AFM1 when lactating animals especially cows ingest contaminated feed continuously and have suggested an increase in AFM1 due to *Staphylococcus aureus* infection and other bacterial infections related with somatic cells diseases (Masoero *et. al.,* 2007; Veldman *et. al.,* 1992). However, little research has been conducted on the transfer of AFM1 into milk as a result of a single consumption of aflatoxin B1. High milk yield and an early stage of lactation have been identified as the main factors contributing to increased carry-over (Masoero *et al.,* 2007; Veldman *et al.,* 1992). Cows in early lactation (2 to 4 weeks after calving) show highest milk yields and a higher carry-over rate than cows in late lactation (34 to 36 weeks after calving), when milk yield naturally declines (Veldman *et al.*, 1992).

Additional factors that have been shown to affect the carry-over rate (often in individual cows) are species difference, general health of the animal, hepatic biotransformation capacity, rate of ingestion and the integrity of the mammary alveolar cell membranes

(Battacone *et al.,* 2003; Fink-Gremmels, 2008). A previous study showed that milk concentration of AFM1 peaked two days after an oral administration in cows (Whitlow *et al.,* 2000). The same study showed that AFM1 disappeared 4 days after AFB1 was removed from the diet. It has been shown that animals must ingest less than 50g and 25g AFB1 per day to comply with the European regulatory levels of contamination in milk set at 0.05 and 0.025 $\mu g/l$ of milk for adults and infants, respectively (Petterson, 1998). Thus cows must ingest less than 10 and 5 kg of feed contaminated at the maximum authorized level (5 g AFB1/kg feed for dairy cattle) to maintain a safe level of AFM1 in milk.

The occurrence of AFM1 in the processed milk was low 3/35 (8.6%) and was contributed mainly by fermented milk (16.7%) and yoghurt (25.0%) (Table 10); their maximum values being above the WHO/FAO threshold limit of $0.05 \mu g/l$ (Table 11). There was no statistically significant difference in the level of contamination between fermented (processed) milk and yoghurt. There was no evidence of contamination in fresh processed milk and UHT. The low incidence and AFM1 levels in processed milk products could be attributed to the low number of samples collected and analyzed from the study area where most of the residents consume raw milk and traditionally fermented milk products (mursik). These results highlight the necessity of a survey involving a larger number of processed milk and milk products. Other studies have showed no AFM1 detected in processed milk samples examined in various countries, such as Argentina Japan and Turkey (Lopez *et al.,* 2003; Tabata *et al.,* 1993; Oruc & Sonal,

2001). In the analysis of UHT milk samples for AFM1 residues, it was found that only 3.7% out the examined samples contaminated with aflatoxin M1 (Alborzi *et al.,* 2006; Unusan, 2006). In a study that investigated the occurrence of AFM1 in UHT commercial milk in Portugal it was shown that only two UHT-treated milk exceeded the set limit while the other 68 UHT-treated samples had low levels (Martins & Martins, 2000). A previous study that investigated 316 sterilized milk samples found that, 0.65% out of examined samples was contaminated with AFM1 (Nachtmann *et al.*, 2007).

The various treatment effects of raw milk may have also lowered the concentration of aflatoxin M1 in the processed samples. According to studies on the effect of various heat treatments on AFM1 content of cow's milk, it was reported that sterilization of milk at 121˚C for 15 min caused 12.21% degradation of AFM1, whereas boiling decreased AFM1 by 14.50% (Choudhary *et al.,* 1998). It was concluded that destruction of AFM1 depends on time and temperature combination of the heat treatment applied. Another study observed that pasteurization caused a decrease in the level of AFM1 at the rate of 7.62% (Sinha, 1998). Other studies showed that pasteurization can partially reduce the amount of AFM1 in milk (Deveci, 2007). However, contradictory data regarding reduction of AFM1 concentration with various heat treatments is available in the literature. Some reports have shown that aflatoxins are stable during heat-treatments such as pasteurization and sterilization (Govaris *et al.,* 2001).

The effect of fermentation was assessed by (Govaris *et al.,* 2002). It was reported that AFM1 levels in all yoghurt samples showed a significant decrease from those initially present in milk. This could be attributed to factors such as low pH, formation of organic

acids or other fermentation by-products, and even to the presence of lactic acid bacteria (Prandini *et al.*, 2009). The mechanism of aflatoxin removal by lactic acid bacteria is still unknown, however, it has been suggested that aflatoxin molecules are bound to bacterial cell wall components rather than metabolically degraded (El-Nezami *et al*., 1998; Haskard *et al.*, 2001; Lahtinen *et al.*, 2004). It was suggested that AFB1 is bound to bacteria through weak non-covalent interactions such as association with hydrophobic pockets on the bacterial surface (El Khoury *et al.*, 2011). The low pH during fermentation alters the structure of milk proteins such as the casein leading to formation of yoghurt coagulum (Govaris *et al.*, 2001). The change in casein structure during yoghurt production may affect the association of AFM1 with this protein causing adsorption or occlusion of the toxin in the precipitate. Up to 34% reduction of AFM1 concentration in yoghurt samples compared to original raw milk samples has been previously reported. It therefore appears that fermentation process of milk could be a practical approach to reduce the risk of this toxin.

Considering the fundamental role of milk and milk products in human nutrition, especially for children, AFM1 in milk and dairy products is considered a significant risk to food safety and public health (Cucci *et al.*, 2007). It is therefore essential to keep feeds of lactating cows free from AFB1 contamination in order to produce AFM1 free high quality milk.

5.2 Conclusions

The results of this study show that the raw cow's milk produced and marketed in the study area has fungal contaminants. The fungal isolates were classified and grouped into 7 genera. *Candida* and *Fusarium* were the most and the least isolated genera respectively. Processed fresh milk and milk products however, had no fungal contaminants.

According to this study, milk (raw, fresh processed, UHT) and milk products (fermented milk, yoghurt) produced in Bomet County are contaminated with aflatoxin M1. The difference in contamination levels between processed and raw milk samples was statistically significant, ($p<0.0001$).

From the results of this study it is evident that the processed milk and milk products are of good mycological quality and have low levels of aflatoxin M1. Raw milk samples, however, are of poor mycological quality and highly contaminated by aflatoxin M1. In regard to this study, processed milk and milk products are therefore safe for consumption as compared to raw milk.

5.3 Recommendations

1. Although majority of farmers clean the cows' udder, milking utensils, there is need for improved hygienic practices to ensure milk free of fungal contaminants.
2. Monitoring programs should be established and be more extensive with a particular attention to monitoring aflatoxin M1 in milk and milk products.
3. There is urgent need to raise awareness on the health effects of chronic exposure to aflatoxin M1 and on the effective detoxification processes.

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APPENDICES

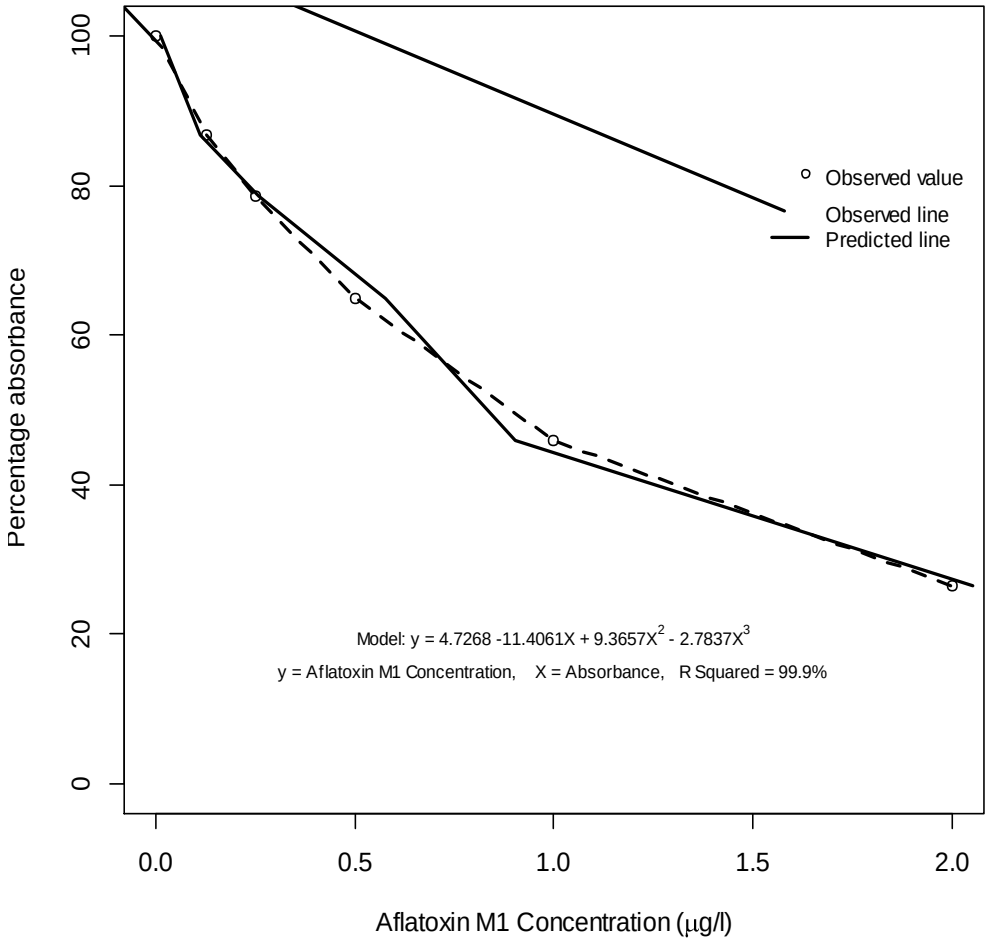
Appendix 1: Macro and microscopic morphology of isolated yeast and yeast-like fungi from milk

Morphology on SDA with chloramphenicol Agar	Appearance on Chromagar Candida	Microscopic Morphology on Cornmeal Agar	Identification by API 20C AUX
Cream to white, pasty; smooth	Greenish blue	Pseudohyphae, true hyphae, round blastoconidia, terminal chlamydospores	*C. albicans*
White to cream, smooth	Light pink	Spherical to ovoid, budding blastoconidia	*C. famata*
Small, round, pasty smooth, white to cream	Purple	Small, uniform in size, and more compactly arranged	*C. glabrata*
Flat, moist, smooth, cream to yellow	Pink to purple	Pseudohyphae; clusters of blastospores	*C. guilliermondi*
Smooth, creamy, and soft	Pink	Elongate blastoconidia along pseudohyphae in a "logs in stream" arrangement	*C. kefyr*
White to cream, smooth, flat	Purple	Branched pseudohyphae with elongated to ovoid blastoconidia,	*C. krusei*
Wrinkled, smooth, creamish white	Purple	Elongate blastoconidia, hyphae and pseudohyphae present	*C. lipolytica*
Smooth, glistening, cream colored	Pink to lavender	Branched pseudohyphae, abudant elongate blastoconidia	*C. lusitinae*
White to cream colored, smooth	Cream to off-white	Abundant much-branched pseudohyphae radiating from a foci, blastospores	*C. parapsilosis*
White, pasty with a blue hue	Light brown	Yeasts with bunch-like, short pseudohyphae bearing blastoconidia	*C.rugosa*

Appendix 2: Morphologic and microscopic variation of isolated molds from milk

Characteristics	*Aspergillus fumigatus*	*Aspergillus niger*	*Aspergillus versicolor*	*Fusarium sp.*
		Morphologic characteristics of the colony		
Surface color	Green to dark green	Dark brown to black	Greyish green	Pink with white margin
Reverse color	Colorless to yellow	Colorless to pale	Reddish brown	Pale
Rate of growth	Rapid	Rapid	Moderate	Slow
Margins	Entire	Entire	Entire	Entire
		Microscopic characteristics		
Hyphae	Branched septate	Branched septate	Septated	Septate
Conidiophore vesicle	Dome shaped	Globose	Spoon-shaped	Short, swollen
Conidial heads color	Bluish green	Blackish brown	Variable	Colorless
Phialides	Uniseriate (covering upper portion of the vesicle).	Biseriate (covering entire vesicle)	Biseriate (cover half to entire vesicle)	Short and swollen
Conidia	Round	Round	Round	Macroconidia slightly Curved

Appendix 3: Enzyme-linked immunosorbent assay calibration curve



Appendix 4: Informed consent (English)

Study title: Mycological quality and aflatoxin M1 contamination of milk and its products in Bomet County.

Institutions and investigators:

Researchers	Institution	Role on Project
Gladys Langat	JKUAT/ ITROMID	Principal investigator
Dr. Christine Bii	KEMRI	Co-investigator
Prof. Viviene Matiru	JKUAT	Co-investigator

Introduction

My name is Gladys Langat, a Master's student at Jomo Kenyatta University of Agriculture and Technology. I am the principle investigator in this study that aims at assessing the mycological quality and aflatoxin M1 contamination of milk and its products in Bomet County. You are being asked to participate in this study because you are eligible. The interview will last approximately 10 minutes only. You can ask any questions you have at any time. This is a consent form that gives you information about the purpose, procedure, risks, benefits, confidentiality/privacy and the process that will be expected during the study. If you agree to take part, please sign your name at the bottom of this form.

Purpose of the study

The purpose of this study is to determine the mycological quality and aflatoxin M1 contamination of milk in the county.

Procedure of study

If you decide to participate in this the study, you will be asked questions that regards milking practices, milk storage, types of feeds given to the cows, etc.

Voluntariness

Participation in this study is voluntary. If you choose not to participate in this study or to leave the study during the interview process, you may do so freely by informing the researcher, without any consequences against you.

Risks of study participation

There are no risks involved in this study. The information that you will provide cannot be identified as belonging to you.

Benefits of participating in the study

You may get no direct benefit from the information you provide for this study. However, the information you provide will be used to assist in formulating policies that may lead to improvement in the quality of milk and milk products in the County.

Study costs

There will be no costs to you for participating in this study apart from your precious time.

Confidentiality

The information in the questionnaire cannot be identified as belonging to you. You will not be personally identified in any publication about this study.

Contacts and Questions

If any time during the project, you have questions about the study, you may contact,

The Secretary,		The Principal Investigator,
KEMRI Ethics Review Committee	or	Gladys Langat
P.O Box 54840-00200, Nairobi.		Mobile: 0723 700 414
Tel: 020-2722541, 0722-205 901, 0733-400 003		Email: *tetiobeto@gmail.com*

Your statement of consent and signature

The above information has been read and explained to me. I have asked questions and received answers. I consent voluntarily to participate in this study. You will be given a copy of this signed form to take with you.

……..	……..
Participant's name	Signature/thumb print and date
……	……
Interviewer's name	Interviewers' signature and date
……	……..
Researcher's name	Researcher's signature and date

Appendix 5: Questionnaire

Reference…….Collection Centre…….

Tick (√) in the box accordingly.

Where do you obtain fresh milk? Own cows ☐ Buy ☐

Where do you milk your cows? Roofed crush ☐ In the open ☐ Under a tree ☐

Do you clean cows' udder before milking? Yes ☐ No ☐

If yes how do you clean the udder? Using cold water ☐ Using warm water ☐ Using detergents ☐

How do you clean your containers? Using hot water ☐ Using cold water ☐

Do you use detergents while cleaning your containers? Yes ☐ No ☐

Where do you store your milk containers? Inside the living house ☐ Maize store ☐

Where do you store your milk? Maize store ☐ Living house ☐

Do you cover the containers containing the milk you have milked? Yes ☐ No ☐

What do you use to feed your cows? Spoilt grains ☐ Napier grass ☐ Grass in the fields ☐

Do you use commercial feeds to feed your cows? Yes ☐ No ☐

If yes where do you obtain them from and how do you store …..……

……

Can I buy a cup of the milk? Yes ☐ No ☐

Appendix 6: Standard Operating Procedures for microscopy techniques and stains

Lactophenol cotton Blue.

For the staining and microscopic identification of fungi

Reagents	Measurements
Cotton Blue (Aniline Blue)	0.05g
Phenol crystals (C6H5O40)	20g
Glycerol	40ml
Lactic acid (CH3CHOHCOOH)	20ml
Distilled water	20ml

This stain was prepared over two days.

On the first day, dissolve the cotton Blue in the distilled water and leave overnight to eliminate insoluble dye.

On the second day, wearing gloves add the phenol crystals to the lactic acid in a glass beaker. Placed on magnetic stirred until phenol is dissolved. Add the glycerol.

Filter the cotton blue and distilled water solution into the phenol/glycerol/lactic acid solution mix and store at room temperature.

Stock Preparation

Chloramphenicol (100mg/ml stock)

To make 10ml stock

Measure 1g of Chloramphenicol then fill to 10ml with 100% ETOH and store in fridge if possible.

Appendix 7: Standard Operating Procedures for media preparation

Sabouraud Dextrose Agar with Chloramphenicol

Sabouraud Dextrose Agar with Chloramphenicol is a modification of Sabouraud Dextrose Agar, with the addition of Chloramphenicol to increase selectivity against commensal microorganisms. It is used for cultivating pathogenic and commensal fungi and yeasts.

Reagents	Measurements
Sabouraud Dextrose agar (oxoid) powder	65g
Chloramphenicol	0.05 g
Distilled water	1000ml

Preparation

1. Suspend 65 g of the medium in one liter of purified water.

2. Heat with frequent agitation and boil for one minute to completely dissolve the medium.

3. Autoclave at 121°C for 15 minutes, remove and pour to plates as required.

CHROMagar Candida Media

It is an isolation and identification medium for *Candida albicans, C. tropicalis* and *C. krusei* from clinical specimens. It is inhibitory to bacteria and can also be used as a selective isolation medium for other yeast species and for filamentous fungi.

Reagents	Measurements
Agar	15g/l
Peptone	10.2g/l
Chromogenic mix	22g/l
Chloramphenicol	0.5µl

Preparation

According to quantities desired, weigh out power and use in the proportion 44.7g/l of purified water ,or use full pre-weighed dose with corresponding volume of water.

Dispense powder slowly in water by rotating until swelling of the agar. Bring water to boil at 100ºC by repeated heating, swirling or stirring regularly. If using an autoclave, do so without pressure. Do not heat to more than 100ºC. Mixture may also be brought to boil in microwave oven; stir gently, return to oven for short repeated heating. Continue until complete fusion of agar grains (Large bubbles replacing foam: about 2 minutes).

Cool to 45ºC, swirling or stirring gently to homogenize before pouring into sterile Petri dishes or tubes. Let dry (Store in dark). Medium may be kept for one day at room temperature or for at least a month in a refrigerator if properly prepared and properly stored. Streak and incubate for 48 hours at 30ºC to 37ºC.

Cornmeal Agar

This medium is useful for stimulating the formation of pseudohyphae true hyphae, arthrospores and chlamydospores in those species able to produce them.

Reagents	**Measurements**
Agar	2g
Distilled water	1l
Cornmeal extract	2g

Heat to dissolve.

Autoclave at 121ºC for 15 minutes.

Appendix 8: API 20c aux yeast identification system.

API 20C AUX is a system for the precise identification of the most frequently encountered yeast in clinical microbiology.

Composition of the Strip

TESTS	ACTIVE INGREDIENTS	QTY (mg/cup.)
0	**None**	**-**
GLU	**D-GLUcose**	**1.2**
GLY	**GLYcerol**	**1.2**
2KG	**calcium 2-Keto-Gluconate**	**1.2**
ARA	**L-ARAbinose**	**1.2**
XYL	**XYL D-XYLose**	**1.2**
ADO	**ADOnitol**	**1.2**
XLT	**XyLiTol**	**1.2**
GAL	**D-GALactose**	**1.9**
INO	**INOsitol**	**2.36**
SOR	**D-SORbitol**	**1.2**
MDG	**Methyl-DD-Glucopyranoside**	**1.2**
NAG	**N-Acetyl-Glucosamine**	**1.2**
CEL	**D-CELlobiose**	**1.2**
LAC	**D-LACtose (bovine origin)**	**1.2**
MAL	**D-MALtose**	**1.2**
SAC	**D-SACcharose (sucrose)**	**1.2**
TRE	**D-TREhalose**	**1.2**
MLZ	**D-MeLeZitose**	**1.2**
RAF	**D-RAFfinose**	**1.9**

Preparation of the strip

Prepare the incubation box (tray and lid) and distribute about 5 ml of distilled water or demineralized water (or any water without additives or chemicals which may release gases (e.g. Cl2, CO2, etc.) into the honeycombed wells of the tray to create a humid atmosphere.

Record the strain reference on the elongated flap of the tray. (Do not record the reference on the lid as it may be misplaced during the procedure.)

Remove the strip from its individual packaging and place it in the incubation tray.

Preparation of the inoculum

Open an ampule of API Suspension Medium (2 ml) or an ampule of API NaCl 0.85 % Medium (2 ml). Using a pipette, pick up a portion of a yeast colony either by suction or by successive touches. Prepare a suspension with turbidity equal to 2 McFarland. This suspension must be used immediately after preparation.

Open an ampule of API C Medium and transfer approximately 100 µl of the previous suspension into it. Gently homogenize with the pipette, avoiding the formation of bubbles.

Inoculation of the strip

Fill the cupules with the suspension obtained in the ampule of API C Medium. Avoid the formation of bubbles by placing the tip of the pipette against the side of the cupule. Care should be taken not to overfill or underfill the cupules (the surface should be flat or slightly convex, but never concave), otherwise incorrect results may be obtained.

Place the lid on the tray and incubate at 29°C ± 2°C for 48-72 hours (± 6°hours).

Reading and Interpretation

Reading the strip

After 48 hours of incubation, or 72 hours (if the tests, in particular glucose, are not clear cut after 48 hours), compare growth in each cupule to the 0 cupule, which is used as a negative control. A cupule more turbid than the control indicates a positive reaction to be recorded on the result sheet.

Morphology test

Determine the presence of hyphae (mycelium) or pseudohyphae (pseudomycelium) using RAT Medium [Rice Agar Tween].

Dispense 1 drop of the suspension obtained in the ampule of API Suspension Medium or API NaCl 0.85 % Medium onto RAT Medium or follow the manufacturer's recommendations. This test constitutes the 21st test of the strip. It is considered positive if hyphae or pseudohyphae are detected.

Interpretation

Identification is obtained with the numerical profile.

Determination of the numerical profile: On the result sheet, the tests are separated into groups of 3 and a number 1, 2 or 4 is indicated for each. By adding the numbers corresponding to positive reactions within each group, a 7-digit number is obtained which constitutes the numerical profile.

Identification

Look up the numerical profile in the list of profiles.