

**Mycotoxigenic Fungi and Mycotoxin Contamination of Traditionally
Fermented Milk (Mursik) in Soliat Location Kericho County, Kenya.**

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**A thesis submitted in partial fulfillment for the Degree of Master of
Science in Molecular Medicine in the Jomo Kenyatta University of
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DECLARATION

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

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DEDICATION

I dedicate this work to my mother; Mrs. Sarah Tanui, my brothers and sisters for their moral and financial support they gave me through the period of my study.

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LIST OF ABBREVIATIONS AND ACRONYMS

A.	<i>Aspergillus</i>
ACH	Acetaldehyde
AFB1	Aflatoxin B1
AFM1	Aflatoxin M1
API	Analytical Profile Index
Bt	beta tubulin
C.	<i>Candida</i>
CFU	colony forming units
CMA	Corn meal agar
DNA	Deoxyribonucleic acid
DON	Deoxyvanelol
E.	<i>Escherichia</i>
EU	European Union
F	<i>Fusarium</i>
FAO	Food and Agriculture Organisation
FDA	Food and Drug Administration
IARC	International Agency for Research on Cancer
ITS	internal transcribed spacer
KEMRI	Kenya Medical research Institute
OSCC	Esophageal squamous cell cancer
PCR	Polymerase Chain reaction
Ppb	parts per billion
PPE	Personal Protective Equipment
ppt	parts per trillion
rpm	revolution per minute
SD	Standard Deviation
spp.	Species

USA	United States of America
USAID	United States Agency for International Development
WHO	World Health Organisation
YPDA	yeast potato dextrose agar

DEFINITION OF TERMS

<i>Amasi</i>	Traditionally fermented milk of South Africa and Zimbabwe.
<i>Kule naoto</i>	Traditionally fermented milk among the Maasai community.
<i>Kwerionik</i>	Ugandan traditionally fermented milk
<i>Mala</i>	Kiswahili reference of fermented milk produced commercially.
<i>Mursik</i>	Traditionally fermented milk among the Kalenjin community.
<i>Nono</i>	Traditionally fermented milk prepared from unboiled cow milk in Nigeria.
<i>Sameel</i>	Traditionally fermented milk of Saudi Arabia.
<i>Senna</i> <i>didymobotrya</i>	Plant used by Kalenjin community of Kenya to treat calabashes and milk before the spontaneous fermentation.
<i>Suusac</i>	Traditionally fermented milk among Somalis of Kenya.
<i>Wara</i>	White soft non-ripened cheese processed by the addition of a plant extract (<i>Calotropis procera</i>).
<i>Wo'sek</i>	Ashes obtained from <i>Senna didymobotrya</i> used in treating calabashes before use in milk fermentation.

ABSTRACT

Mursik is traditional fermented milk. Fungi are among the major contaminants of milk including mycotoxigenic fungi. Mycotoxigenic fungi may grow in *mursik* and produce mycotoxins that can cause poisoning to consumers. This study aimed to enumerate fungal species contaminants including fungi responsible for mycotoxin production and quantification of the mycotoxins. Microbiological analysis was done on 194 samples from Soliat Location and 4 samples from commercial outlets where fungal enumeration was carried out on Potato Dextrose Agar. Polymerase Chain Reaction (PCR) was used to detect moulds and mycotoxins extracted using Enviroligix procedure and quantified using quicktox kit. Data was analyzed using SPSS version 17 for descriptive statistics. Yeast species isolated from *mursik* samples were: *Geotricum candidum* 124 (32.56%) as the predominant strain, *Rhodotorula* species 19 (4.99%), *Sacharomyces cerevisiae* 13 (3.41%), *Candida parapsilosis* 41 (10.76%), *Candida albicans* 22 (5.77%), *Candida tropicalis* 67 (17.59%), *Candida glabrata* 53 (13.91%) and *Candida krusei* 21 (5.51%) and moulds were of *Aspergillus*, *Penicillium* and *Fusarium* genus. Aflatoxin was detected in 196/198 (99.0%) of the samples where levels ranged between 2-12 parts per billion exceeding the required levels of 0.05ppb. Fumonisin toxin was detected in only 3 (1.5%) of the samples mean of 0.008ppb and Deoxynivalenol toxin was detected in 1 (0.5%) sample with the level of 0.001. Both fumonisin and Deoxynivalenol levels were below the standard levels. Eighty percent of the *mursik* producers milk their cows in the open and store their milk and their milk fermenting calabashes at a maize store and/or in the living rooms which are risk factors for contamination of *mursik* with mycotoxigenic fungi and other microorganisms. Microorganisms isolated are responsible for mycoses and mycotoxins cause mycotoxicoses. These results will help the authorities to develop measures to tame the contamination of milk and milk products.

CHAPTER ONE

1 INTRODUCTION

1.1 Background Information

In Kenya, dairy industry is increasingly becoming a smallholder farmers' domain presently, owning over 80% of the 3 million heads of dairy cattle. It contributes to approximately 56% of the total milk production and 80% of the marketed milk. Similarly, in many third world countries, livestock rearing and their products is an important part of the national economy. Subsistence and semi-commercial smallholder farming systems are dominated by resource constrained farm households (Lanyasunya *et al.*, 2005).

Milk fermentation is a very common practice in Africa. In South Africa, fermented milk known as *Sethemi* is made from naturally fermenting milk. Among the Maasais', fermented milk is known as *Kule naoto* and *Suusac* by the Somalis of Kenya while it's known as *Amasi* in both South Africa and Zimbabwe (Chelule *et al.*, 2010). Among the Kalenjin community in Kenya, fermented milk is known as *Mursik*. It is fermented from raw and boiled milk and prepared in calabashes (gourds) where the calabashes are first treated with charcoal like material obtained from burning plant called *Senna didymobotrya*. The milk is treated with the same and left to ferment naturally for three to four days (Mureithi *et al.*, 2000). The production of fermented milk in Kenya and other African countries does not involve the use of starter cultures, suggesting that the fermentation arises spontaneously from microbes originating from the environment, processing equipment, or processors (Eyassu *et al.*, 2012).

Milk forms favorable medium for growth and multiplication of microorganisms because of its high nutrient content. It contains proteins, carbohydrates, minerals and vitamins which support the growth of many forms of fungi and yeasts. These include species from the genus, *Aspergillus*, *Penicillium*, and *Candida* (Abd El- Aziz *et al.*, 2011). Thus, besides its beneficial effects on nutrition, milk though can also act as a vehicle for the transmission of diseases of bacterial (like brucellosis, tuberculosis, salmonellosis, listeriosis), viral (like

hepatitis, foot-and-mouth-disease), rickettsia (Q-fever) or parasitological (toxoplasmosis, giardiasis) origin. Milk is an excellent culture and protective medium for certain microorganisms, particularly bacterial pathogens, whose multiplication depends mainly on temperature and competing microorganisms and their metabolic products. Where milk is produced under poor hygienic conditions and is not cooled, the main contaminants are usually lactic acid producers which cause rapid souring. Lactic acid has an inhibitory effect on pathogenic bacteria but this cannot be depended upon to provide a safe milk product (Heeschen, 1994).

According to Saadia (2010), *Aspergillus spp.*, *Alternaria spp.*, *Fusarium spp.*, *Neurospora spp.* are the most common fungi species found in milk products. Other documented milk contaminants includes: *Aspergillus glaucus*, *A. niger*, *Alternaria spp.*, *Cheotomium candidum*, *Cladosporium herbarum*, *Fusarium spp.*, *Monilia spp.*, *Mucor rouxii*, *Neurospora spp.*, *Penicillium expansum*, *Penicillium spp.*, *Rhizopus nigricans*, *Sporotrichum carinis* and *Thamnidium elegans*. Fungi of the genus, *Fusarium*, *Aspergillus*, *Penicillium*, *Cladosporium*, *Geotrichum* and *Cladosporium species* have been isolated in milk during different seasons in different geographical locations (Pešić-Mikulec *et al.*, 2005). Most of these fungi have been known to be mycotoxin producers (Uzeh *et al.*, 2006).

With the numerous species of fungi present in milk with mycotoxic potential, there is a possibility that there will be contamination with multiple toxins (Whitlow *et al.*, 2005). Use of available feed resources is further complicated by farmers' inability to use them before they spoil especially during wet season. Colonization of molds in wet season results in mycotoxin risk mainly associated with favorable climatic conditions, poor feed handling and storage practices. Mycotoxins are passed to milk when the cows are fed with contaminated feeds and also fungi responsible for these toxin productions can colonize milk thus producing significant amounts of toxins under poor handling practices (Lanyasunya *et al.*, 2005).

According to Kang'ethe and Lang'a (2009), in a study carried out to determine the level of aflatoxin in milk in urban towns, thirty five percent (in Nairobi, Nakuru, Nyeri, Eldoret and

Machakos) 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. A larger number of people consume fresh and fermented milk in the rural areas. No report has been documented on the mycotoxin contamination of the milk in these areas which poses a health risk. Additionally, fermented milk undergoes a process which is traditionally practiced, providing favorable conditions for milk contamination with mycotoxin producing fungi. Thus, the objective of this study was to enumerate fungal contaminants including fungi responsible for mycotoxin production and quantification of the mycotoxins in *mursik* collected in Soliat Location.

1.2 Statement of the Problem

Mursik is a common diet among the Kalenjin community. Fungi and other microbes can colonize fermented milk since they can survive a wide range of temperatures and fermentation. Warm humid conditions of 18°C-37°C, poor livestock feeds and conditions of storing milk and milk products handling favour the growth of mycotoxigenic fungi. The formation of mycotoxins in nature is considered a global problem though in certain geographical areas of the world, some mycotoxins are produced more readily than others (Lawlor & Lynch, 2005). In most European countries aflatoxins are not considered to be a major problem. However, vomitoxin, ochratoxin, Zearalenone are found more frequently. Aflatoxins are common in Asian and African countries and certain parts of Australia. In a study done in Iran, where detection of Aflatoxin M1 (aflatoxin metabolite) in Iranian milk, showed that 80% of the samples had AFM1 greater than the maximum tolerance limits (0.05ppb) accepted by the European Union and Iranian national standards (Tabari, 2010). In United States, 10% excess of primary liver-cell cancer was observed in the Southeast, where the estimated average daily intake was high (IARC, 1993). In Kenya, Kang'ethe and Lang'a (2009), indicated that 99% of pasteurized market milk was contaminated with aflatoxin M1 where 31% of the contaminated samples exceeded the WHO/FAO levels of 0.05ppb. Contamination of mycotoxins will lead to mycotoxicoses in consumers.

1.3 Justification

Owing to its high nutrient content milk is a good culture media for microorganisms. Fermentation of milk allows the growth of some microbes while inhibiting the growth of others. Research has shown that fungi can colonize fermented milk since they can survive a wide range of pH and temperatures. There was therefore need to research on fungal contaminants in *Mursik* as these can survive processes like boiling done to freshly retrieved milk from the cows before fermenting. Also, to determine the presence of mycotoxins as these may be produced by fungal contaminants or passed along the food chain through consumption of contaminated feeds. Fungal contamination is accelerated by poor milk handling methods and storage of animal feeds. Mycotoxins pose a health hazard to public health and no research has been done in Kenya to determine the presence of fungal and mycotoxin contaminants in *Mursik*. The rise in chronic diseases including cancer may be associated with mycotoxins exposure. The study site has all the favorable conditions for fungal growth and people ferment milk traditionally and practice mixed farming. Isolation of fungi responsible for mycotoxins has been done majorly in fresh milk. Aflatoxin is one of the most widely occurring and dangerous mycotoxins and is the most studied toxin (C.A.S.T. 1989). Thus this study included the study of other mycotoxin like, fumonisin, vomitoxin by quantifying them because they have been known to cause illnesses in both humans and animals. The study focused on quantifying mycotoxins in traditionally fermented milk (*Mursik*) and detects mycotoxigenic fungi responsible for mycotoxin production which has not been conducted by the fore researchers. This is essential given the public health implication of mycotoxin exposure and the risk of carcinogenic, hepatotoxic and mutagenic potential of mycotoxins (Riley *et al.*, 2001). This study aimed to enumerate fungal species contaminants including fungi responsible for mycotoxin production and quantification of the mycotoxins.

1.4 Research questions

- i. What are the types of fungi contaminating traditionally fermented milk (*mursik*) from Soliat Location, Kericho County?
- ii. What are the types of mycotoxins contaminating the milk and their levels?

- iii. Which genes coding for mycotoxigenic fungi are present in fermented milk from Soliat location; Kericho County?

1.5 Hypothesis

Null hypothesis: Traditionally fermented milk (*mursik*) is contaminated with mycotoxigenic fungi and mycotoxins.

1.6 Objectives

1.6.1 General Objective

To investigate mycotoxigenic fungi and mycotoxin contamination of fermented milk produced in Soliat location, Kericho County.

1.6.2 Specific Objectives

1. To identify fungi from fermented milk (*mursik*) from Kericho county.
2. To detect genes of fungi responsible for mycotoxin production in *mursik*.
3. To establish the level of aflatoxin, fumonisins and deoxynivalenol contamination in the fermented milk (*mursik*).

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Mursik

Spontaneous fermentation and preferential selection of those foodstuffs whose sweetness was improved by this process, remains the origin of consumable fermented products (Stiles, 1996) and allowed our ancestors to keep foodstuffs at ambient temperature. Fermented milk was first produced around 10'000 years ago (Tamime, 2002) when the Neolithic Revolution transformed hunters and gatherers into settled land-based communities. The first evidence that cattle, sheep and goats were domesticated for milk production dates back to 5000 BC (Teuber, 1995). The actual origin of fermented milks is unknown but there is no doubt that their consumption dates back to prehistoric times (Helferich & Westhoff, 1980). Milk fermentation is dating to 1300 BC cited in the Old Testament (Roginski, 1988). Cheese and butter consumption by the general population in the ancient Kingdom of Meroe (690 BC – AD 323) is recorded in detail (Abdelgadir *et al.*, 1998).

In many third world countries, Kenya included, livestock rearing and their products is an important part of the national economy (Lanyasunya *et al.*, 2005). In regard to its high nutrient content, milk form a favourable habitat for growth and multiplication of microorganisms. Milk supports the growth of many forms of fungi and yeasts. These include species from the genus *Aspergillus*, *penicillium*, and *Candida* (Abd El- Aziz *et al.*, 2011). Fermentation is a method of food preservation, used from old age. The traditional practice of fermenting milk products is common in Asia, Africa, the Middle East, and Northern and Eastern Europe (Savadogo *et al.*, 2004). For more than 330 years ago, it became part of the cultural and traditional norm among the indigenous communities mostly in the third world nations, majorly in Africa (Chelule *et al.*, 2010; Abeer *et al.*, 2009). Traditional fermentation is a way of food processing, where microbes, lactic acid bacteria for example, *Lactobacillus*, *Lactococcus*, *Streptococcus* and *Leuconostoc* species are used.

Fermentation of milk mainly involves lactic acid bacteria, but micrococci coryneforms, yeasts and moulds can be involved (Zamfir *et al.*, 2006; Ogier *et al.*, 2004; Hassan *et al.*, 2006). In lactic acid (non-alcoholic) fermentation, lactic acid is the main by-product of the fermentation process, the pH of the ferment is always lower than 5. Foods processed using fermentation process include: beverages, dairy products, cereals and even meat products (Chelule *et al.*, 2010).

In general, fermented food products constitute a major portion of the daily diet in Africa (Jespersen, 2003; Sanni, 1993). The use and production of fermented milk is an old tradition among Kenyans. Every tribe has their own unique methods of producing fermented milk, and the differences between fermented milks are based on the location of tribal communities and the different production processes. Fermentation of milk is an ancient practice of man which has been passed down from generation to generation. The aim of fermenting was to obtain products with characteristic flavor, aroma and consistency and at the same time, could be stored unspoiled for a longer time than untreated raw milk (Chelule *et al.*, 2010).

In Kenya, traditional fermented milk products are mainly produced by pastoral communities such as the Maasai, Borana, Kalenjins, Gusii and Somali. They are mainly produced by spontaneous fermentation of the milk in traditional containers such as gourds and skin bags. Some of these fermented milks have been reported to have health beneficial properties such as *Kule naoto* produced by the Maasai, *mursik* produced by the Kalenjin and *ambere amaruranu* produced by the Gusii (Mathara *et al.*, 2008; Mathara *et al.*, 1995; Mokuu, 2004). *Mursik* is mainly produced by the Kalenjin community in Kenya, through spontaneous fermentation of cow milk in a traditionally prepared gourd (Mathara *et al.*, 1995; FAO, 1990). It forms a major part of the Kalenjin diet due to its delicious taste and belief that it improves health (Mathara *et al.*, 1995). The Kalenjin also value it as a special drink that is shared in special occasions to symbolize success of certain activities such as successful marriage negotiations and weddings, victory in athletics among other events.

In the Kalenjin tribe the fermented milk, *mursik*, is produced in specific calabash gourds, also known as *sotet*. *Sotet*, a traditionally treated gourd is made from the hollowed out dried

fruit of the plant *Lagenaria siceraria*. Some days before the milk is treated, a small branch of an *Ite or Senetwet* tree (*Senna didymobotrya*) is cut and allowed to dry. One end of this tree is first burned in a fire and a dried, cleaned gourd is rubbed on the inner surface gently with it. A chopped end stick from the tree *Olea africana* locally known as Enkidogoe in Maasai and Sosiot in Kalenjin is used to break charcoal inside (Mathara, 1999). This is repeated several times until the gourd is fully coated inside with charcoal dust. This process reduces the porosity of the gourd and improves the flavor. The gourd is filled with milk which is also treated with charcoal from *Senna dydimobotrya* tree and then closed by a special cap obtained from a cow hide and left to ferment naturally by microbes from the environment or those pre-exist in milk. Repeated use of naturally occurring microbiota to inoculate fresh milk, commonly referred to back-slopping, has been the developmental keystone of traditional fermented milk products worldwide (Wouters *et al.*, 2002). After fermentation, the product is gently shaken before consumption. Some Kalenjin groups also add small quantities of blood obtained from prickling a vein in the neck region of a healthy bull, and from which fibrin has been removed by gentle stirring. Addition of blood can impact the microbial metabolism, as iron is an important cofactor for a number of essential cellular processes. A thick bluish layer forms on the surface when mursikis ready. It is shaken well before drinking, to ensure that a uniformly thick emulsion is formed. In some Kalenjin households, fermented milk is consumed several times daily (Mikko *et al.*, 2012).

Fermentation and all of these additions to the process are used to improve the odor, the taste, and the flavor of fresh milk. In an interview with farmers, Mureithi and colleagues noted that farmers were of the opinion that fresh milk smells and tastes like cow urine and had to be improved before it can be consumed. Also, lack of refrigeration, and the need to store milk for the dry season (when milk production decreases due to a lack of pasture) required that excess milk be stored for a longer time. For example, the Pokot developed *chekha mwaka*, specially treated milk that could be stored for more than a year without getting spoiled (Mureithi *et al.*, 2000).

Some of the herbs have been known suitable for the purpose of imparting the preservative and aromatic effect to milk. Some of the plant herbs used by Kalenjins in Kericho County

include *sertwet* (*Acacia meansii*), *simotwet* (*Ficus thoningii*), *suriat* (*Rhus natalensis*), *muokiot* (*Lippia kituiensis*), *Olea Afrikana* (*emitiot*) with a majority preferring to use *Senna didymobotrya* (*senetwet*), mostly citing its ease of availability (Wangila *et al.*, 2014). Products of *S. didymobotrya* used in the treatment of calabashes and fresh milk have been known to be medicinal among the Kalenjin community. In Kenya, traditionally Kipsigis community has been using these plants to control malaria as well as diarrhea. In addition, they use to treat skin conditions of humans and livestock infections as well. *S. didymobotrya* is useful in the treatment of fungal and bacterial infections (Raja, 2012).

Mathara *et al.*, 1995 and Nakamura *et al.*, 1999, established that *Mursik* culture consist of lactic acid bacteria (LAB) species which include *Lactococcus lactis subsp. lactis*, *Leuconostoc mesenteroides subsp. dextranicum*, *Lb. curvatus*, *Leuc. paramesenteroides* and *Lb. planturum*. The local dairy products produced and widely consumed by Fulanis in Nigeria are *Nono* and *wara*; *Nono* is prepared from unboiled cow milk collected in a calabash and allowed to ferment naturally while *Wara* is a white soft non-ripened cheese processed by the addition of a plant extract (*Calotropis procera*) to the non-pasteurized whole milk from cattle. The nature of fermented products varies from one region to another. It depends on the local indigenous microflora, which in turn reflects the climatic conditions of the area (Savadogo *et al.*, 2004). Studies have shown that; *Aspergillus niger*, *Aspergillus fumigatus*, *Penicillium chrysogenum*, *Rhizopus spp.*, *Fusarium moniliforme*, and *Trichoderma reesii* are the fungi mostly isolated from the two products (Uzeh *et al.*, 2006; Abeer *et al.*, 2009).

Fermented dairy products for example, yoghurt, cheese and sour milk are protein-rich milk products. They have been acclaimed both by popular believe and some research findings as being more nutritious and health promoting than fresh milk. Reports by Platt (1964) stated that fermented milk is a good source of the B vitamins, including vitamin B12. There are also claims that the digestibility of the milk proteins is improved by fermentation (Marshal, 1986). Yoghurt, a fermented milk product, has the ability to kill pathogens and build the immune system against invading organisms. People of Finland consume large amounts of yoghurt and it has been suggested that they harbor numerous intestinal lactobacilli that have anticarcinogenic properties (O' Sullivan *et al.*, 1992). According to Mathara *et al.*,

1995 some of the LAB has antimicrobial properties against pathogenic bacteria species such as *Staphylococcus aureus* and *Salmonella typhimurium*.

Lactobacillus bacteria have been known to produce exopolysaccharides (EPS), components which contribute to the beneficial health effects. These exopolysaccharides are excreted by microorganisms onto the surface of their cell walls as cohesive layers or into the growth medium as slime (ropy) exopolysaccharides. A wide variety of microorganisms including moulds, yeasts, bacteria and algae have been reported to produce these EPS (Kumar *et al.*, 2007). EPS have been reported to contain properties such as oligofructans, glucooligosaccharides and β -glucan are prebiotic or bifidogenic thus boosting health to the consumers (Badel *et al.*, 2011; Korakli *et al.*, 2003). EPS has other health beneficial effects which include reduction of blood cholesterol levels, anti-carcinogenic, anti-temporal and immunomodulation activity (Ruas and Reyes, 2005). LAB EPS enhance the attachment and colonization of the gut by probiotics and this antagonizes the effects of pathogenic microorganisms. *Lactobacillus rhamnosus* RW-9595M produces EPS which increase production of the cytokine IL-10 by the macrophages, and these prevent development of inflammatory conditions (immunesuppression) in the gut (Lebeer *et al.*, 2010; O'connor *et al.*, 2005).

2.2 Mursik and its contaminants

Fermentation does not take place in controlled systems or sterilized conditions; as a result contamination with yeasts and some pathogenic bacteria may arise, thus making fermented milks to become major vehicles of transmission for many food borne pathogens. Food borne diseases caused by these pathogens were problematic and must have continually preoccupied early man (Uzeh *et al.*, 2006). Unhygienic milking procedures and equipment used for milking, filtering, cooling, storing or distributing milk is also an important source of microorganisms. This situation is aggravated if the equipment is not properly cleaned and sanitized after use. Milk residues left on equipment and utensil surfaces provide nutrients to support the growth of many microorganisms, including pathogens (Bryan, 1983). Poor hygiene, practiced by handlers of food products, may lead to introduction of pathogenic microorganisms into the products and since they do not undergo further

processing before consumption, these foods may pose risk to consumers (Kang'ethe & Lang'a, 2009).

Fermented products have been reported to exhibit the highest incidence and level of yeast contamination due to the low pH which forms a selective environment for their growth (Vogel *et al.*, 2002). Hence, different yeast species have been isolated from fermented dairy products (Mathara *et al.*, 2004). Their presence is due to various properties such as fermentation or assimilation of lactose, production of extracellular proteolytic and lipolytic enzymes, assimilation of lactic acid and citric acid, growth at low temperatures and tolerance to elevated salt concentrations (Akalin *et al.*, 2006).

Yeasts are widely distributed in nature and are therefore often found as contaminants in both commercial and traditional fermented milk and have been enumerated and identified in a wide variety of African fermented food products including milk (Fleet and Mian, 1987; Fleet, 2007; Jespersen, 2003). The presence of yeasts indicates that they are able to proliferate during milk fermentation and positively react with LAB (Pereira-Dias *et al.*, 2000; Gadaga *et al.*, 2001). In yoghurt, their occurrence is mainly a consequence of the contamination and hence they are a major cause of yoghurt spoilage (Fleet, 1990). However, in traditionally fermented milk, yeasts are part of the indigenous microflora, coming into the product with the raw milk or from the environment and containers (Gadaga *et al.*, 2000).

The yeast species frequently found in dairy products include *Debaryomyces hansenii*, *Candida famata*, *Kluyveromyces marxianus*, *Candida kefir*, *Candida stellata*, *Saccharomycopsis lipolytica*, *Saccharomyces cerevisiae*, *Candida krusei*, *Rhodotorula glutinis* and *Rhodotorula rubra*. However, *D. hansenii* and *Kluy. marxianus*, as well as their asporogenous equivalents *C. famata* and *C. kefir* respectively, emerge as the most prevalent ones (Fleet, 1990). Al-Otaibi, (2012) isolated *Candida lusitania*, *Cryptococcus laurentii*, *Saccharomyces cerevisiae* and *Candida kefir* Sameel, traditionally fermented milk in Saudi Arabia. *Saccharomyces cerevisiae*, *Candida Lusitania*, *Candida colliculosa* and *Saccharomyces dairenensis* were the predominant strains in isolated Zimbabwean traditional fermented milk (Gadaga *et al.*, 2000). Abdelgadir *et al.* (2001), in Sudan and

Shuangquan *et al.*, (2006) in Mongolia, found that the predominant yeast strains in the fermented milk were *Sacharomyces cerevisiae* and *Candida kefyr*.

The yeast isolated in *susaac*, Kenyan traditionally fermented camel were, *Candida krusei*, *Geotricum penicillatum* and *Rhodotorula muciloginosa* (Lore *et al.*, 2005). This shows that different yeast species were predominant in different fermented milk products. Moulds which have been known mainly in milk and cheese fermentation and include, *Penicillium*, *Mucor*, *Geotrichium*, and *Rhizopus* species. Uzeh *et al.*, (2006), isolated moulds of the species, *Aspergillus niger*, *Aspergillus fumigatus*, *Penicillium chrysogenum*, *Rhizopus spp*, *Fusarium moniliforme*, and *Trichoderma reesii* in *nono* and *wara*, traditionally fermented milk products of Nageria. The fungi *Aspergillus*, *Penicillium*, *Rhizopus*, *Fusarium*, and *Trichoderma* species which were isolated are identified spore formers, which therefore mean that they can easily contaminate the dairy products which are usually exposed during processing, storage, and hawking. The fungi of the genus *Aspergillus*, *Penicillium*, *Fusarium* have been among the contaminants of raw milk and are major spoilage organisms of carbohydrate foods (Alborzi *et al.*, 2005; Rhodes & Fletcher, 1966). However, their growth can result in the production and accumulation of mycotoxins which are of public health and economic importance.

2.3 Mycotoxigenic fungi

Mycotoxigenic fungi are mould which produce mycotoxins as their secondary metabolites that exert toxic effects on animals and humans. The toxic effect of mycotoxins on animal and human health is referred to as mycotoxicosis (CAST, 2003). Mycotoxigenic fungi are readily isolated from diverse environmental samples, soil and plant tissues or residues, are considered the natural habitat of these fungi (Cotty, 2004). Soil serves as a reservoir for primary inoculum for the contamination (Abbas *et al.*, 2006). Mycotoxigenic fungi has been enabled by their phenotypic and metabolic flexibility to colonize a broad range of agriculturally important products and to adapt to a range of environmental conditions. They are structurally diverse, deriving from a number of biosynthetic pathways and their effect upon consumers is equally diverse ranging from acutely toxic to immunosuppressive or carcinogenic (Moretti *et al.*, 2013).

Colonization of mycotoigenic fungi on foods, affects the quality of food, their organoleptic attributes, and nutritional quality. Fungi same as other microorganisms will assimilate and utilize the most readily available nutrients in the materials they grow upon thus resulting in food spoilage (Cegielska-Radziejewska *et al.*, 2013). Fungi of the specis *Aspergillus niger*, *Aspergillus fumigatus*, *Penicillium chrysogenum*, *Rhizopus spp*, *Fusarium moniliforme*, and *Trichoderma reesii* has been known to contaminate fermented milk of all kinds at different geographic locations (Uzeh *et al.*, 2006; Pesic-Mikulec *et al.*, 2005). Moulds of genera, *Aspergillus*, *Penicillium*, *Rhizopus*, *Fusarium*, and *Trichoderma* species which were isolated in traditionally fermented milk *nono* of Nigeria were identified as spore formers, which therefore mean that they can easily contaminate the dairy products which are usually exposed during processing (Uzeh *et al.*, 2006). Species that belong to the genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* have been considered the most toxic which produce mycotoxins such as aflatoxins, zearalenone, T2-toxin, deoxynivalenol, ochratoxin A, fumonisins, and patulin the most common mycotoxins found in animal feeds and food (Pitt & Hocking, 2009; Hussein & Brasel, 2001). Mycotoxin production is a complex secondary metabolite process (Geiser *et al.*, 1998).

In the recent past, molecular techniques have increased the possibilities to characterize milk microbial ecology. Amplification of specific DNA fragments using polymerase chain reaction (PCR) and specific probes is extensively sensitive and has the potential to detect the presence of fungi in agricultural commodities (Manonmani *et al.*, 2005). The use of PCR to identify mycotoxin fungi is attracting considerable attention (Haughland *et al.*, 2004).

Genotyping techniques have shed new light on mycotoxin producing fungi and provided the foundation for advances in detection methodology. Historically, fungi have been identified on the basis of traditional taxonomic characteristics (for example, morphological features); more recently, the tools of molecular biology have enabled genetic analysis and classification on the basis of nucleic acid sequence. Since analytical methods for detecting mycotoxins have become more prevalent, sensitive, and specific, surveillance of foods for mycotoxin contamination has become more common (Michealsen *et al.*, 2006).

Internal Transcribed spacer (ITS) regions contain the most conserved sequence at the terminal region and also contain the hyper variable sequences distinguishing between species. The use of ITS region as compared with other molecular probes is advantageous due to many reasons including increased sensitivity because of existence of more than 100 copies per genome (Mirhedi *et al.*, 2007).

Until recently, the molecular identification of fungi to species level has been based mostly on the use of variable internal transcribed spacer (ITS) regions. The non-coding ITS regions have a high copy number in the fungal genome as part of tandemly repeated nuclear rDNA. These regions benefit from a fast rate of evolution, which results in higher variation in sequence between closely related species, in comparison with the more conserved coding regions of the rRNA genes (Michealsen *et al.*, 2006).

As a consequence, the DNA sequences in the ITS region generally provide greater taxonomic resolution than those from coding regions (Lord *et al.*, 2002; Anderson *et al.*, 2003). In addition, the DNA sequences in the ITS region are highly variable, divergent, and distinctive, and might serve as markers for taxonomically more distant groups (Michealsen *et al.*, 2006).

The coding portions of many fungal 18S, 5.8S and 28S rDNA genes are greatly preserved and primers to these regions have been generated (Brown *et al.*, 2001). These allow the isolation of the internal transcribed spacer sequences, which lie between the coding regions, from a wide range of fungi. The ITS region is amplified from the target fungus and polymorphism within the ITS region is generally at the level of species, rather than between isolates of the same species, making it an ideal target for the development of species-specific PCR assays (Nule, 2001).

The tubulin gene family consists of three major highly conserved sub-families, alpha-, beta-, and gamma-tubulin, which arose from a series of gene duplications in early eukaryotic evolution (Keeling & Doolittle, 1996). The tubulin genes are made up of highly conserved proteins which are the principle structural and functional components of eukaryotic microtubules that are major components of the cytoskeleton, mitotic spindles, and flagella of eukaryotic cells (Thon & Royse, 1999).

Several studies have made use of beta-tubulin genes to examine relationships among fungi at all levels, and has been found to be a useful tool in both deep level phylogenetic studies and studies of complex species groups (O'Donnell *et al.*, 1998a). Studies that have used the beta-tubulin gene for characterization of *Fusarium* species include Geiser *et al.*, (2001), Mach *et al.*, (2004), Reischer *et al.*, (2004), and Yli-Mattila *et al.*, (2004). Geiser *et al.*, (2001) used the beta-tubulin gene, as well as the *EF-la* gene, to identify and characterize *F. hostae*. Mach *et al.*, (2004) employed the beta-tubulin gene for the early and specific detection of *Fusarium langsethiae*, and distinguishing it from related species of section *Sporotrichiella*. Yli Mattila *et al.*, (2004) demonstrated that the beta-tubulin gene was able to distinguish between *Fusarium poae*, *Fusarium sporotrichioides*, *Fusarium langsethiae* and *Fusarium kyushuense*.

2.4 Mycotoxin genes and toxin synthesis

Mycotoxin production is a complex secondary metabolite process. Most of the specific enzymatic activities required for mycotoxin production are encoded in a gene cluster but additional unlinked loci also are required (Geiser *et al.*, 1998). Aflatoxins are toxic secondary metabolites produced by a 70-kb cluster of genes in *Aspergillus flavus*. The cluster genes are coordinately regulated and reside as a single copy within the genome (Carrie *et al.*, 2007). Jiujiang *et al.*, (1995) showed that at least nine genes involved in the aflatoxin biosynthetic pathway are located within a 60-kb DNA fragment.

The genes, *nor-1*, *aflR*, *ver-1*, and *omtA*, have been cloned in *A. flavus* and *A. parasiticus*. In addition, five other genes, *pksA*, *uvm8*, *aad*, *ord-1*, and *ord-2* have been cloned in *A. parasiticus*. The *pksA*, *aad*, and *uvm8* genes exhibit sequence homologies to polyketide synthase, aryl-alcohol dehydrogenase, and fatty acid synthase genes, respectively. The *ord-1* and *ord-2* genes involved in later steps of aflatoxin biosynthesis, have been determined; the *ord-1* gene product exhibits homology to cytochrome P-450-type enzymes. Order of aflatoxin pathway genes within 60-kb DNA region has been found to be *pksA*, *nor-1*, *uvm8*, *aflR*, *aad*, *ver-1*, *ord-1*, *ord-2*, and *omtA* in *A. parasiticus* and *nor-1*, *aflR*, *ver-1*, *ord-1*, *ord-2*, and *omtA* in *A. flavus*. The order is related to the order in enzymatic steps required for aflatoxin biosynthesis (Jiujiang *et al.*, 1995).

Significant progress in understanding of fungal secondary metabolism include the discovery of sterigmatocystin (ST) biosynthetic gene clusters and the discovery of a G-protein-mediated growth pathway in *A. nidulans* regulating secondary metabolism production (Tag *et al.*, 2000; Brown *et al.*, 1996). According to Barnes *et al.* (1994) sterigmatocystin is a highly toxic intermediate in the AFB1 biosynthetic pathway. AFB1 biosynthetic pathway in *A. flavus* and *A. parasiticus* and the ST biosynthetic pathway in *A. nidulans* are believed to be similar. It is now apparent that structural genes required for secondary metabolite production are usually clustered and that the regulation of the clustered genes is largely dependent on pathway-specific transcription factors (Tsuji *et al.*, 2000; Keller & Hohn, 1997).

Linkage of aflatoxin pathway genes was first evidenced in an *A. parasiticus* cosmid clone, NorA that contains both *nor-1* and *ver-1* genes (Skory *et al.*, 1992). A physical and transcriptional map of the 35-kb genomic DNA insert in cosmid NorA suggested that several genes involved particularly in the early stages of aflatoxin B1 biosynthesis are clustered on one chromosome in *A. parasiticus*. These include the *pksA* gene, which codes for a polyketide synthase, the *nor-1* gene, which codes for a reductase that converts norsolorinic acid to averantin, the *ver-1* gene, which is involved in the conversion of versicolorin A to sterigmatocystin and the *omtA* gene, coding for an S-adenosyl methionine-dependent O-methyltransferase that converts sterigmatocystin to O-methyl sterigmatocystin and dihydrosterigmatocystin to dihydro-O-methylsterigmatocystin (Chang *et al.*, 1992; Skory *et al.*, 1992; Cleveland *et al.*, 19987). The *omtA* gene has been cloned and characterized for both *A. parasiticus* and *A. flavus* (Yu *et al.*, 1993). In addition to these structural genes, a regulatory gene, *aflR* (previously named *afl-2* for *A. flavus* and *apa-2* for *A. parasiticus*), that codes for a regulatory factor (Aflr protein) has been cloned and was shown to be involved in the activation of pathway gene transcription (Chang *et al.*, 1993).

The step for the conversion of O-methyl sterigmatocystin and dihydro-O-methylsterigmatocystin to AFB1 and AFB2 appears to be catalyzed by an oxidoreductase enzyme complex, which may consist of two or more subunits. Genes that code for the polypeptides of this enzyme complex have not yet been characterized. In addition, a

putative fatty acid synthase gene, *uvm8*, potentially involved in polyketide backbone synthesis, first identified by complementation of a UV mutation and a gene, *aad*, homologous to aryl-alcohol dehydrogenase potentially involved in an intermediate step of aflatoxin biosynthesis have been cloned (Jiujiang *et al.*, 1995).

Aspergillus nidulans, produce the mycotoxin sterigmatocystin (ST), which also serves as the penultimate precursor in the aflatoxin (AF) biosynthetic pathway (Cole & Cox, 1981). Both AF and ST are among the most toxic, carcinogenic and mutagenic compounds. Study of the *A. nidulans* ST pathway has led to identification of a 60 kb gene cluster (the ST Cluster; *stc*) that includes 25 co-regulated genes, many of which have been shown to function in ST biosynthesis (Brown *et al.*, 1996). Transcription of all of these genes is dependent upon the activity of *aflR*, a pathway-specific regulatory gene found within the ST cluster that encodes a zinc binuclear cluster type DNA binding protein (Woloshuk *et al.*, 1994). *aflR* expression is regulated during the life cycle such that *aflR* mRNA begins to accumulate early in the stationary phase and activation of other genes required for ST biosynthesis quickly follows (Yu *et al.*, 1996a).

All fumonisin biosynthetic (*FUM*) genes characterized to date are localized within a 42.5 kb region of the *F. Verticillioides* genome (Proctor *et al.*, 2003). The clustering of genes involved in the biosynthesis of secondary metabolites in filamentous ascomycetes is common. A fumonisin biosynthetic gene (*FUM*) cluster has been described in species *Fusarium proliferatum*, *Fusarium verticillioides* and one of the rare fumonisin-producing strains (FRC O-1890) of *F. oxysporum* and in the more distantly related fungus *Aspergillus niger* (Proctor *et al.*, 2008; Khaldi & Wolfe, 2011). The *Fusarium* cluster includes 16 genes that encode biosynthetic enzymes as well as regulatory and transport proteins. Each selected *FUM* gene (*FUM1*, *FUM3*, *FUM6*, *FUM7*, *FUM8*, *FUM10*, *FUM13*, *FUM14* and *FUM19*) is required for wild-type fumonisin production in *F. verticillioides*, and together they span almost the entire length of the *FUM* cluster (Alexander *et al.*, 2009). The PM genes included eight genes (*CAL1*, *CPRI*, *HIS3*, *RED1*, *RPB1*, *RPB2*, *TEF1* and *TUB2*) that have been used previously to infer phylogenetic relationships within *Fusarium*; two global regulatory genes, *LAE1* and *FLB2*, that also affect fungal secondary metabolism and a

dehydrogenase gene, *ZBD1*, that flanks the *FUM* cluster in *F. verticillioides* (Proctor *et al.*, 2003; Proctor *et al.*, 2009; Wiemann *et al.*, 2010).

Functions of most of the genes in fumonisin biosynthesis in *F. verticillioides* have been determined by gene inactivation and heterologous expression analyses. Within the cluster, the *FUM1* gene encodes a polyketide synthase that catalyses synthesis of a linear polyketide that forms the backbone structure of fumonisins. In addition, the *FUM8* gene encodes an α -oxoamine synthase that mediates whether fusaria produce FBs or FCs by catalysing the condensation of the linear polyketide with alanine to produce FBs or with glycine to produce FCs. The number, order and orientation of genes within *FUM* clusters in *F. verticillioides*, *F. oxysporum* and *F. proliferatum* are the same (Proctor *et al.*, 2008).

Many of the Deoxynivalenol (DON) biosynthesis genes are localized in a gene cluster of at least 10 genes. The genes in this cluster include those for trichodiene synthetase (*tri5*), P450 oxygenase (*tri4* and *tri11*), acetyltransferase (*tri3* and *tri7*), transcription factors (*tri6* and *tri10*), a toxin efflux pump (*tri12*), and two unidentified hypothetical proteins (*tri8* and *tri9*). Another acetyltransferase gene (*tri101*) is unlinked to the cluster (Kimura *et al.*, 1998). Recently, two *F. sporotrichioides* genes, *tri13* and *tri14*, were found to be under the control of *tri10*, but the functions of these genes are unknown. Homologs of *tri* genes have been reported for *G. zeae* (Tag *et al.*, 2001). Lee *et al.*, (2001), analyzed the sequences of *tri* genes from *G. zeae* DON chemotypes and reported that, of the 10 *tri* gene homologs in the *tri* gene cluster, all except *tri7* were conserved.

tri5 encodes a trichodiene synthase that catalyses the first committed reaction in the trichothecene biosynthetic pathway. The nucleotide sequence of the *tri5* gene has been characterized in several *Fusarium* species (Hohn & Desjardins, 1992). The *tri6* gene encodes a protein that regulates the trichothecene biosynthesis genes and has been sequenced in *F. sporotrichioides* (Proctor *et al.*, 1995).

2.5 Mycotoxins

2.5.1 Mycotoxin Contamination in milk

Mycotoxins are secondary metabolites of fungi that are toxic. The word Mycotoxin plainly means poison from fungi. Amid the thousands of species of fungi, only about 100 are known to produce mycotoxins. There are three major genera of fungi that produce mycotoxins: *Aspergillus*, *Fusarium* and *Penicilium*. Although between 300 and 400 mycotoxins are known, those mycotoxins of most concern, based on their toxicity and occurrence, are aflatoxin, deoxynivalenol (DON) or vomitoxin and fumonisin (Lanyasunya *et al.*, 2005).

Mycotoxins are responsible for mycotoxicoses in animals and humans. The expression of toxicity in animals is diverse since different fungal species produce different compounds (Tarik *et al.*, 2005). Cattle feed is at a risk of contamination from activities of microbes like yeast and fungi invading the feeds and producing mycotoxins, toxic compounds which pose threat to the health of dairy cattle. Cattle feed basically comprises of cereals mixed with green fodder. Cereals form very good substrates for fungal growth such as: *Aspergillus*, *Penicillium* and *Fusarium* species and susceptible to mycotoxin contamination (Sultana & Hanif, 2009). Maize can be contaminated by mycotoxins such as aflatoxins, ochratoxin A, trichothecenes, and fumonisins (Wang & Liu, 2006; Sangare-Tigori *et al.*, 2006; Pietri *et al.*, 2004; Nikiema *et al.*, 2004; Pietri *et al.*, 2004; Arino *et al.*, 2007).

Mycotoxins are not only accumulated in muscles of all animal species, but through metabolism it is excreted in urine and faeces. It is also found in eggs of poultry and animal milk. Attention must be paid to lactating animals with regard to the possible excretion of metabolites in milk. The mean rate of presence in milk varies according to the mycotoxins' minimum levels which range from 0.3-2.2% for AFB1 to 0.05% for FB1 and T2-toxin. Ochratoxin A and Vomitoxin residues can only be found in cow's milk when high quantities of toxins have been administered to animals. Occurrence of AFM1 in milk is a matter of concern in relation to the transfer of mycotoxins in the dairy food chain (Whitlow *et al.*, 2005).

Several mycotoxin contamination interventions measures are recommended including: good storage practices, addressing food shortages, public mycotoxicological food safety and educational campaigns on dangers of mycotoxin exposure. This multiple approach is not only a cost effective way of reducing mycotoxin exposure but it also attracts long term sustainability (Kang'the & Lang'a, 2009).

Effective mycotoxin detection and control strategies have eliminated mycotoxicosis in developed countries (Guo *et al.*, 2000). These strategies include; inclusion of sorbent materials in feed or addition of enzymes or microorganisms capable of detoxifying mycotoxins has been reported to be reliable methods for mycotoxin prevention in feeds, also by incorporating pro-biotics or lactic acid bacteria into the diet (Jard *et al.*, 2011; Hell & Mutegi, 2011). However, while bentonite and aluminosilicate clays have been used as binding agents for reducing aflatoxin intoxication in cattle without causing digestive problems when mixed with aflatoxin-contaminated feed (Diaz *et al.*, 1997). Devegowda and Castaldo (2000) found that using glucomannan supplementation at 0.05% of diet of dairy cows that consumed aflatoxin-contaminated feed; there was a reduction of 58% in aflatoxin in the cow's milk. While in developing countries prevention of mycotoxins from entering the food chain may not currently be receiving sustainable attention or focus as in developed countries because of different food systems, financial constraints, availability of food policies, levels of food safety education and technological development posing as some of the challenges (Adegoke & Letuma, 2013).

2.5.2 Effects of Mycotoxin Contamination

Fungal metabolites are toxic to humans and farm animals when concentrations are higher than 5ppb (parts per billion) FAO/WHO limits (Kang'ethe & Lang'a, 2009; Bennett & Klich, 2003). Mycotoxicoses are diseases caused by mycotoxins and are examples of "poisoning by natural means" and thus are analogous to the pathologies caused by exposure to pesticides or heavy metal residues. The symptoms of a mycotoxicosis depend on the type of mycotoxin; the amount and duration of the exposure; age, health, and sex of the exposed individual; and many poorly understood synergistic effects involving genetics, dietary status, and interactions with other toxic compounds. Thus, the severity of mycotoxin

poisoning can be compounded by factors such as vitamin deficiency, caloric deprivation, alcohol abuse, and infectious disease status. In turn, mycotoxicoses can heighten vulnerability to microbial diseases, worsen the effects of malnutrition, and interact synergistically with other toxins (Calderone *et al.*, 2002).

Mycotoxins cause a variety of adverse effects in humans ranging from allergic responses to immunosuppression and cancer (Pitt, 2000). Mycotoxins are responsible for a decrease in breeding, production and subsequent economic losses for farmers (Hussein & Brasel, 2001). The occurrence of these fungal metabolites may also be of public health concern. Some mycotoxins are now linked with the incidence of certain types of diseases and have brought global concern over feed and food safety, more so for milk and milk products. Aflatoxin B1 (AFB1) which is also known as Aflatoxin M1 after metabolic breakdown of aflatoxin B1 and can be detected in the milk of lactating cows which have consumed significant quantities of aflatoxin B1 (Lanyasunya *et al.*, 2005). Aflatoxin B1 has been known to be carcinogenic in humans leading to hepatocarcinoma (IARC, 1993). Aflatoxin M1 cannot be denatured through pasteurization or in yoghurt and cheese processing (Chelule *et al.*, 2010). Ochratoxin A is probable carcinogen causing urinary tract cancer and kidney damage in humans while fumonisins appear to be the cause of oesophageal cancer in man (Marasas *et al.*, 2001). Acute mycotoxin poisoning results in the weakening of liver or kidney functions, in severe cases results in deaths (Pitt, 2000).

The majority of mycotoxicoses, result from eating contaminated foods. Skin contact with mold infested substrates and inhalation of spore-borne toxins are also important sources of exposure. Except for supportive therapy (diet, hydration), there are no treatments for mycotoxin exposure, although Fink-Gremmels, (1999) described a few methods for veterinary management of mycotoxicoses, and there is some evidence that some strains of *Lactobacillus* effectively bind dietary mycotoxins (El-Nezami *et al.*, 1998)

2.5.3 Mechanism of Action of Mycotoxins

According to Pitt (2000), mycotoxins have diverse modes of action. Some mycotoxins act by interfering with protein synthesis thus resulting in adverse effects ranging from skin

sensitivity or necrosis to extreme immunodeficiency. Aflatoxin B₁ is genotoxic in that, it induces DNA changes hence known as a cancer initiator (Mace *et al.*, 1997). In contrast, AFB₁ does not interact with DNA, but probably modifies the cell death and proliferation mechanisms, acting as a cancer promoter (Riley *et al.*, 2001). The co-contamination of foods by many mycotoxins, with different modes of action raises the problem of a possible synergy amongst these toxic metabolites. That is the reason why the presence of mycotoxins in foods and feeds has evoked regulation in many countries (FAO, 2004).

2.6 Factors Influencing Fungal Growth and Mycotoxin Production in Food

Aspergillus flavus known to produce aflatoxins in corn is favored by heat and drought stress related with warmer climates. In wheat, high moisture at flowering is associated with increased incidence of mycotoxin formation. In corn, *Fusarium* diseases are mostly associated with warm conditions at silking, insect damage and wet situation late in the growing season. *Penicillium* molds grow in wet and cool conditions and some require oxygen. Molds flourish on the surfaces of cheeses where oxygen is present and low pH being selective for them. Molds commonly found growing in vacuum-packaged cheese include *Penicillium spp.* and *Cladosporium spp.* (Loralyn *et al.*, 2009). These are potential mycotoxin producing fungi (Uzeh *et al.*, 2006).

2.7 Aflatoxins

Aflatoxins, are secondary metabolites produced by species of *Aspergillus*, specifically *Aspergillus flavus* and *parasiticus* fungi, which are naturally occurring contaminants of food and elaborate the toxins under favorable conditions of temperature, relative humidity and poor storage conditions. There is high risk of farmers feeding AFB₁contaminated animal feeds to their animals. Aflatoxin M₁ (AFM₁) is the principal hydroxylated AFB₁ metabolite present in milk of cows fed with a diet contaminated with AFB₁ and excreted within 12 hours of administration of contaminated feeds (Kang'ethe & Lang'a, 2009).

Aflatoxins are a family of extremely toxic, mutagenic, and carcinogenic compounds. Two structural types of aflatoxins are known, B and G types, of which aflatoxin B₁ is considered the most potent and mostly found in Kenya during outbreaks (Azziz-Baumgartner *et al.*,

2005; Sherphard, 2003). Toxigenic *A. flavus* species produce aflatoxins B 1 and B2, and toxigenic *A. parasiticus* species produce aflatoxins B 1, B2, G1, and G2. Aflatoxin B1 is a carcinogen and is excreted in milk in the form of aflatoxin M1 (Kang'ethe & Lang'a, 2009).

Exposure to aflatoxins is a result of ingestion of contaminated foods. Ingestion of aflatoxin poisoned food causes hepatic and gastrointestinal injury, immunosuppression, teratogenic, and oncogenic effects (Henry *et al.*, 1999; 2002). Constant exposure to low-level of aflatoxin enhances the danger of hepatocellular carcinoma (Chao *et al.*, 1991). Acute liver injury, morbidity and mortality have been associated with high exposure to aflatoxins. Intake of 2-6 ppb of aflatoxin for a month can result in acute hepatitis leading to death (Patten, 1981). Aflatoxin in swine leads to reduced weight gain, immunosuppression, hepatitis and death (Peraica *et al.*, 1999). The Food and Drug Administration (FDA) has established action levels of 20 parts per billion (ppb) for grain and feed products, and 0.5 ppb for milk. Grain, feed, or milk having aflatoxin at or above these levels cannot be allowed for consumer consumption (FAO, 1997). Recommended limits in feed are: 20 ppb for dairy animals, 100 ppb for breeding cattle, breeding swine, and mature poultry (USAID, 2004).

2.8 Fumonisin

Fumonisin is a secondary metabolite produced by the fungus of the genus *Fusarium*. Fumonisin is responsible for production of trichothecene mycotoxins. Trichothecenes are found mainly on small grain cereals such as wheat, barley, oats and rye, whereas fumonisins are mainly linked with maize. The most common forms of fumonisins are B1, B2 and B3 (Rheeder *et al.*, 2002; Marasas, 2001). Ingestion of these secondary metabolites may cause a range of adverse effects in different animal species. Variety of malignancies in many consumers has been associated with the consumption of fumonisin contaminated maize (Gelderblom *et al.*, 1988).

Ingestion of these secondary metabolites may cause a range of adverse effects in different animal species. Variety of malignancies in many consumers has been associated with the consumption of fumonisin contaminated maize (Gelderblom *et al.*, 1988). Fumonisin has

been known as the causative agent of equine leukoencephalomalacia, a neurotoxicological aberration in horses and pulmonary oedema in pigs (Marasas *et al.*, 1988; Ross *et al.*, 1990). It has been established that fumonisin causes hepatic cancer in experimental mice and rat models (Marasas, 2001). Fumonisin in humans has been related with neural tube defects in people that rely on maize as a staple food, but most prominently it has been indicated as a potential cause of oesophageal cancer in humans (Missmer *et al.*, 2006; Marasas, 2001). In certain regions around the world including Bomet County in Kenya, where high daily intake of maize and maize-derived commodities occurs, an associations between either mouldy maize, *F. verticillioides* or fumonisin and the incidence of oesophageal cancer have been reported (Marasas, 2001; Doko & Visconti, 1994).

The toxic concentrations of fumonisin differ much depending on the animal species (Rheeder *et al.*, 2002). Absorption of about 5 - 10 mg/kg fumonisin in feed induces neurotoxic effects in horses (Marasas *et al.*, 1988). In pigs the ingestion of 4 - 16 mg/kg body weight may result in liver cirrhosis and more than 16 mg/kg body weight result in pulmonary edema (Marasas, 1995). Chickens can withstand higher concentrations of fumonisin in feed, up to 75 mg/kg and cattle seem not to be affected by high fumonisin concentrations (Gelderblom *et al.*, 1991).

The fumonisins are highly water-soluble and unlike all other food mycotoxins because they do not have an aromatic structure or a unique chromophore for easy analytical detection. They are primary amines with 2 tricarballylic groups, which contribute to their water-solubility. Study of metabolic pathways is in progress. Fumonisin synthesis is known to involve acetate precursors and alanine and several of the genes involved in fumonisin synthesis have been identified as a cluster on chromosome 1 in *F. verticillioides* (Desjardins *et al.*, 2000).

2.9 Deoxynivalenol (DON) or Vomitoxin

Deoxynivalenol (DON) is a secondary metabolite produced by a *Fusarium species*. It is one of the most mycotoxin commonly detected in feed. DON is also called vomitoxin because it was first associated with vomiting in swine. DON has can be detected in milk due to contamination of milk by *Fusarium species* (Uzeh *et al.*, 2006) or through

contamination of cattle feeds (Gajecki *et al.*, 2010). Dairy cattle consuming feeds contaminated primarily with DON have led to reduction in milk production and can be detected in milk. Lactating cows are more susceptible to infections caused by feed-borne mycotoxins than beef cattle due to their very high performance levels (Seeling *et al.*, 2006).

In healthy animals, DON is very quickly converted into de-epoxide in the rumen. De-epoxide is far less toxic, which explains the animals tolerance to the presence of this substance in feed. In animals with a previous history of ruminal acidosis, DON is not fully broken down, and its presence is determined in the blood (Seeling *et al.*, 2006). Grass haylage contaminated with deoxynivalenol causes the toxic syndrome in cattle, which is characterized by more pronounced inflammations, enteritis, mastitis and laminitis (Weaver *et al.*, 1980; Whittlow *et al.*, 2005). Research has shown that DON causes polyribosomal breakdown in mammalian cell lines. Cell signaling pathways are activated by 1mg DON, through gene induction and activation of several nitrogen-activated protein kinases. DON activates hematopoietic cell kinase and double-stranded RNA-activated protein kinase, which leads to apoptosis. Numerous studies have shown that DON is immunotoxic in animal models (Briani *et al.*, 2008).

The existence of DON in the gastrointestinal tract encourages the production of mucosal antibodies and autoantibodies (Baumgart & Carding, 2007). DON raises IgA secretion from Peyer's patch lymphocytes through the activation of MAP kinases and proinflammatory cytokines, and *COX-2* gene expression (Pestka & Smoliński, 2005). DON has nephropathic properties, and it causes dysfunctions in IgA secretion (Baumgart & Carding, 2007; Briani *et al.*, 2008). It therefore indicates that when DON is administered in low doses to monogastric organisms; DON increases the production of IgA which interacts with many self-antigens and intestinal bacteria (Maresca & Fantini, 2010).

Human exposure to DON may be within the range of doses shown to be immunotoxic in rodents, human exposures and responses to this toxin are ill defined (Whittlow *et al.*, 2005). Gastrointestinal symptoms including vomiting were apparent in humans after high levels of DON intake in China (Luo *et al.*, 1988). A similar outbreak was observed in India when local villages consumed rain damaged wheat that contained DON and other

trichothecenes (Bhat *et al.*, 1989). Also, along with Fumonisin, DON outbreak was also observed in Transkei, South Africa (Isaacson, 2005)

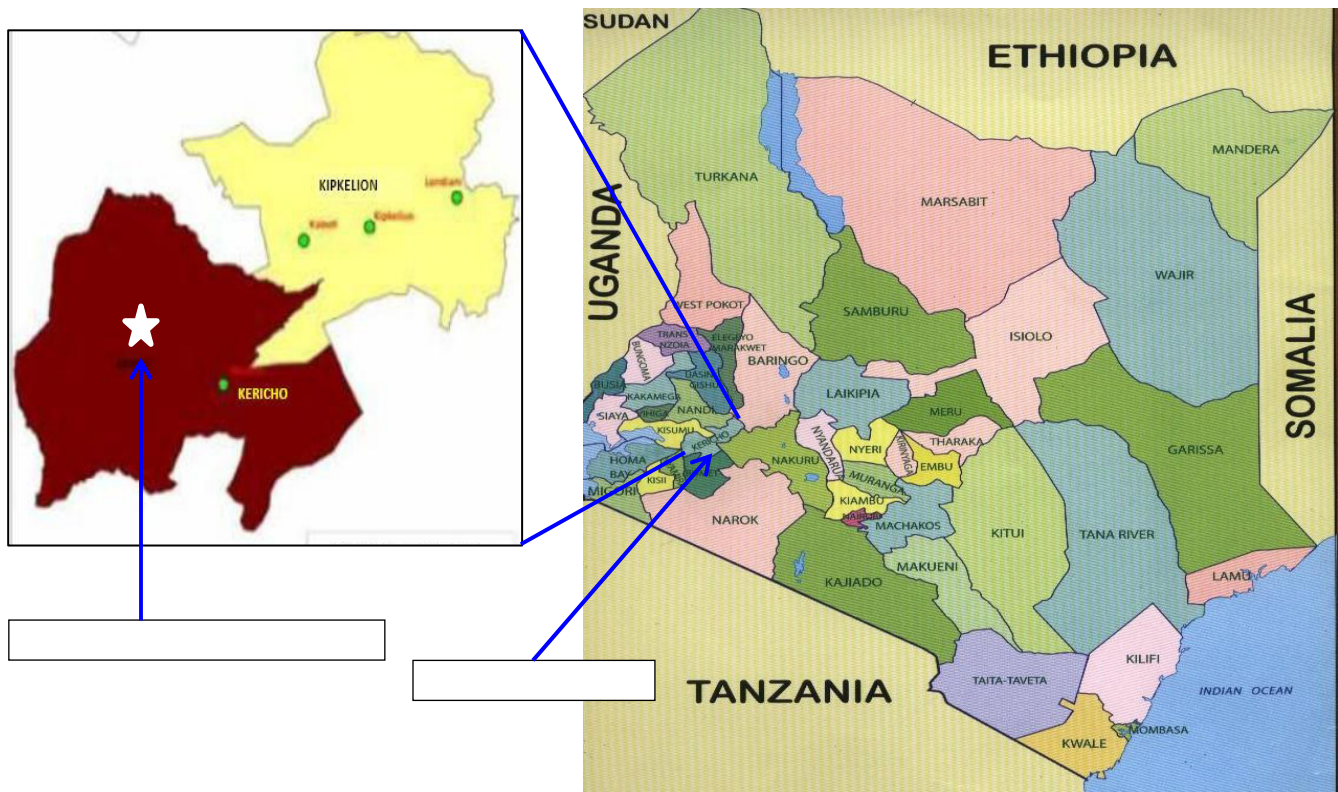
CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Study area

Fermented milk samples were collected from Soliat location, Kericho County Kenya. This area of study consists of many households, where each household has livestock and prepares fermented milk. This area is located at the lower part of Kericho District where its temperatures are of hot and humid; temperature range from 18°C-37°C (Kang'ethe & Langa, 2009), conditions known to favor the growth of mycotoxigenic fungi although there is no documentation in the country showing fungal and mycotoxin contamination in the area (Figure 3.1).

Figure 3. 1 Map showing the area of study (Soliat Location).



3.2 Sample size determination

194 samples were collected from the area of study, assuming rate of 17% of milk is contaminated by fungi and mycotoxins as reported by Kang'ethe and Lang'a, (2009). Prevalence at 5% accuracy.

The following formula by Fisher *et al.*, (1998) was used:

$$N = \frac{Z^2 (P (1-P))}{D^2}$$

Where;

N= sample size

Z= standard error from the mean at 1.96

P= prevalence (17% rate of contamination of milk with mycotoxins)

D= absolute precision (at 5%)

a= level of significance (at 5%)

Hence, $N = \frac{1.96^2 \cdot 0.17(1-0.17)}{0.05^2} = 216.8$ or 217 samples.

This means at 5% accuracy, a minimum of 217 samples was to be collected in the area; however, due to unavailability of *mursik* in the rest of selected (23) households in the area, 194 samples were collected.

3.3 Study design

The study was laboratory based carried out on *mursik* samples collected from households from Soliat Location, Kericho County. It was conducted between February and August, 2013. All *mursik* samples were processed at mycology laboratory, Center for Microbiology Research (CMR), KEMRI.

Sampling was done by using simple random sampling method. The number of households in the area was determined by obtaining the area chief's records; the households were numbered and randomly selected by random numbers.

3.4 Investigation variables

Preparation of fermented milk by use of boiled and raw milk, use of calabashes, use or no use of charcoal like material (Wo'sek) to treat their calabashes before use, the interval of milk addition to their continuous cultures during the period of fermentation, storage of fermenting milk and calabash conditions and washing or no washing of calabashes before the next batch. This information was obtained by administering a questionnaire to the persons from whom *Mursik* was collected (Appendix I).

3.5 *Mursik* Sample collection

According to simple random sampling technique; each household in each sublocation was given a number, mixed and picked randomly. *Mursik* samples were collected in the 4 sub locations. The owner of each household was provided with a sterile, glass a 50ml bottle where they pour the fermented milk in to it, tightly closed with the lid and transported to the laboratory in a cool box, to arrest further fermentation.

3.6 Storage of samples

Samples were stored at 4°C for 72 hours before processing or at -40°C when longer storage was anticipated.

3.7 Safety Measures in the Laboratory

All procedures were carried out in accordance to the general laboratory bio-safety and waste disposal guidelines. These included working under level two biosafety cabinets, wearing of personal protective equipments (PPE) and careful handling of glassware and other tools while working with isolates.

3.8 Mycological Investigations of *Mursik*

Fermented milk samples were collected from Soliat location, Kericho County and cultured for fungal isolation. Briefly, 1ml of *Mursik* sample was suspended in 10ml of sterile water and vortexed for 1 minute. This was repeated in all the samples under investigation. One milliliter of the sample was then inoculated onto Yeast Potato Dextrose Agar (Oxoid)

(YPDA) supplemented with chloramphenicol to inhibit bacterial contamination and incubated at 25° C for 72 hours. After incubation, soft, moist white/cream colonies grew on the plates in 24 to 48 hours and were considered positive. Fungal colonies were sub-cultured onto new YPDA plates for purity. This was done by touching a specific colony using an inoculating applicator sticks and streaking on the new YPDA and incubated for 72 hours at at 25°C (Nakavuma *et al.*, 2011). The identification of fungal colonies was done as described by Larone (2002).

Microscopy was done to determine the micromorphological features of each fungal isolate. Briefly, a small part of each colony was picked and smears of the colonies were made on heat sterilized glass slides. The slides were then flooded with lactophenol blue solution for up to one minute, excess lactophenol from slides was removed and blotted and covered with a cover slip. The slides were examined under the oil immersion lens at x100 (David and Joseph, 2000).

3.8.1 Identification- Chromogenic Agar Candida (ChroMagar)

It is a primary medium for differentiating between various *Candida* species based on their colony colour and morphology. Preliminary identification of yeasts was done by sub-culturing onto ChroMagar candida where each colony was transferred by touching using an inoculating applicator sticks and streaking on the ChroMagar and incubated at 30°C for 72 hours (Fujita *et al.*, 2001).

3.8.2 Analytical Profile Index (API 20C AUX- Biomeriux)

This was used as a confirmatory test for all the *Candida* spp.; it is based on fermentation and utilization of substrates such as glucose, maltose, sucrose and lactose and to observe fermentation of glucose and maltose and absence in sucrose. The basal medium in the ampoules were thawed by placing them in a boiling water bath for two minutes and allowed to cool; an incubation tray was prepared and 20 ml of water dispensed and strip placed into the incubation tray; the ampoules were opened by breaking the glass tip inside its tip, the molten medium was inoculated with two colonies (>2 mm diameter) of the test yeast fungus; the inoculated media was introduced onto the strip

(20 cupules; approximately 0.2 ml each) following the manufacturer's directions; the lid was replaced on the tray and incubated at 28 to 30°C for 72 hours (Karin & Joel, 1993).

Results were read and recorded after 24, 48, and 72 hours of incubation. Growth in each cupule was compared to cupule 0 which was used as negative control. A cupule more turbid than the control was recorded as a positive reaction. Identification was obtained with numerical profile. On the result sheet, the test was separated into groups of three and a number 1, 2 and 4 was indicated for each group sequentially. The numbers corresponding to positive reactions within each group were added to obtain a number which constituted the numerical profile (Appendix III).

3.9 Molecular analysis of Mycotoxigenic fungi

3.9.1 Genomic DNA Extraction

DNA extraction was done by pipetting 500µl of *mursik* into a vial, centrifuged at 3000 rpm for 20 minutes, the supernatant discarded and the concentrate resuspended in 200 µl of sterile distilled water, heated at 100° C for 10 minutes a water bath and again centrifuged to obtain a supernatant. The supernatant was transferred to another vial for use a template in the PCR (Dragan *et al.*, 2010).

3.9.2 Cloning of Fungal beta-tubulin (Bt) and Internal Transcribed Spacer (ITS) using Polymerase Chain Reaction (PCR) procedure

Fungal genes of Beta-tubulin (β t) and Internal Transcribed Spacers (ITS) region of the ribosomal RNA gene were amplified by PCR. Amplification was carried out in 50 µl reaction mixture containing: The β -tubulin gene was amplified and the primers were Bt forward primer Bt2a 5'-GGT AAC CAA ATC GGT GCT GCT TTC-3' and Bt reverse primer Bt2b 5'-ACC CTC AGT GTA GTG ACC CTT GGC-3' which amplified a fragment between 400-500 bp. The 18S – 28S ITS region of ribosomal DNA was amplified using the ITS5 forward primer and ITS4 reverse primer with sequences, 5'-GGA AGT AAA AGT AAC AAG G-3' and 5'-TCC TCC GCT TAT TGA TAT GC-3' respectively, amplifying the region of 600bp. Amplification was performed in a thermocycler in 50 µL

reaction mixtures containing 5µL of 10× PCR buffer, 1µl of dNTP 10mM of each, 2.5 µl of MgCl₂, 1µM of each primer, 1.5 µl of Taq polymerase, and 1µL of DNA template and PCR water to add up to 50 µL. The PCR conditions were: initial denaturation at 95°C for 5min, then 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. Final extension was carried out at 72°C for 7 min, then the amplified product was kept at 4°C (Dragan *et al.*, 2010). Monoplex PCR was used for each primer set as described by Dragan *et al.*, (2010) technique.

Gel electrophoresis was used to detect the presence of the amplified PCR product. A volume of 2µl of PCR products was subjected to electrophoresis on a 5% agarose gel stained with ethidium and viewed under ultraviolet light to detect the presence and size of the amplified DNA product (Dragan *et al.*, 2010).

3.10 Mycotoxin analysis

3.10.1 Mycotoxin Extraction

Aflatoxin

Briefly, 25ml of *Mursik* was measured into a disposable sample cup, 50mL of 50% ethanol was added; the sample cup was capped tightly and shaken using a hand for 2 minutes. The extract immediately separated into two layers. The top yellowish layer containing aflatoxin residues was used in testing. Using a calibrated pipette with a new tip, 100µl of DB4 Buffer provided in the kit was put into a reaction vial. Exactly, 100µl of the top yellowish layer of the sample was then added into the reaction vial containing the buffer. The buffer and sample extract were then mixed thoroughly by drawing the liquids up and down in the pipette tip (<http://www.envirologix.com>).

Fumonisin

Briefly, 25ml of *Mursik* was measured into a disposable sample cup, 50mL of 50% ethanol was added; the sample cup was capped tightly and shaken using a hand for 2 minutes. The extract immediately separated into two layers. The top yellowish layer containing fumonisin residues was used in testing. 2 sets of vials were used for each sample being tested: the first set for dilution, the second set for testing (<http://www.envirologix.com>).

Dilution vial set number 1. Using a calibrated pipette with a new tip, 100 μ L 50% ethanol was pipetted into the dilution vial. Using the same tip 100 μ L from the top yellowish layer of extract was pipetted and added to the dilution vial containing the ethanol and mixed well with pipette by drawing liquids up and down in the pipette tip (<http://www.envirologix.com>).

Dilution vial set number 2. Using a new pipette tip, 100 μ L of DB2 Buffer was added to the (second) reaction vial, used for testing. Using the same tip, 100 μ L of the well mixed diluted sample extract from the dilution (first vial) was transferred into the reaction (second) vial containing the DB2 Buffer, and mixed well by drawing the liquids up and down in the pipette tip until the mixture was uniformly light yellow (<http://www.envirologix.com>).

Deoxynivalenol

Briefly, 10ml of *Mursik* was measured into a disposable sample cup, 50mL of tap water was added; the sample cup was capped tightly and shaken using a hand for 30 seconds. The sample was allowed to settle until two distinct separate layers were visible. The top layer containing DON residues was used in testing. Using a calibrated pipette with a new tip, 0.25 μ l of dilution buffer provided in the kit was put into a reaction vial. Exactly, 150 μ l of the top yellowish layer of the sample was then added into the reaction vial containing the buffer. The buffer and sample extract were then mixed thoroughly by stirring with pipette tip (<http://www.envirologix.com>).

3.10.2 Mycotoxin Quantification

Mycotoxins were quantified using a QuickTox Quicksan kit. QuickTox Strips canisters was removed the refrigerator and allowed to come to room temperature before opening. Quick to strips to be used were removed and the canister resealed immediately. The strips were placed into each reaction vial containing the buffer and the extract with the arrow at the end of the strip pointing into the reaction vial. The sample extract travelled up the strip and allowed to develop for 5 minutes. Immediately the arrow tape of the strip was cut off and discarded and strip inserted into the Quicksan reader for quantification where a strip

was inserted face down in the carrier. The carrier was inserted into the reader and the strips were read by touching or clicking on the “Read Test” area of the screen. Results were recorded in an electronic worksheet (<http://www.envirologix.com>). This procedure was used in the quantification of aflatoxin, fumonisin and Deoxynivalenol.

Results were reported in parts per billion (ppb); the quantification units and the samples which contained less amounts of mycotoxins were reported as <LOD (less than Limit of Detection).

3.11 Data Analysis

The data collected was entered using Ms. Excel, a computer package, and uploaded to statistical computer software, SPSS version 17 and analyzed for descriptive statistics.

3.12 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 2347 (Appendix IV) prior to the study. The participants were recruited randomly where each household in each sublocation was given a number, mixed and picked without discrimination by using the documentation of the local authority; area chief. Consent from individuals was done before sampling their milk by having them read and sign the consent form and for the people who are not able to read, another person who understood the language well read for them to understand what was going on and let them decide if an individual was part of the study without influence. A copy of the consent form remained with the participant. All signed documents were filed and kept away from access by other people apart from the principal investigator (Appendix II). Samples were coded and individuals were not exposed by name or home and a link to the participants was created and kept in different logbooks where the Principal Investigator could access. The collected data were recorded in logbooks which were kept in drawers under lock and key to avoid access by other people. Also the data was stored in a computer where the computer have password known only by the principal investigator.

CHAPTER FOUR

4 RESULTS

4.1 Samples Collected in Four Sub-locations in Soliat Location

A total of 198 *mursik* samples were used in the study. Study sites comprised of 4 sub locations, all within Soliat Location. Of these samples, 30% (59), 27% (53), 22% (44) and 19% (38) were collected from Soliat, Kongeren, Motero and Kamasega sub locations, respectively. In addition, 2% (4), used as controls, these were the fermented milk (*Mala*) were obtained from the commercial shops found in the stated areas (Figure 4.1).

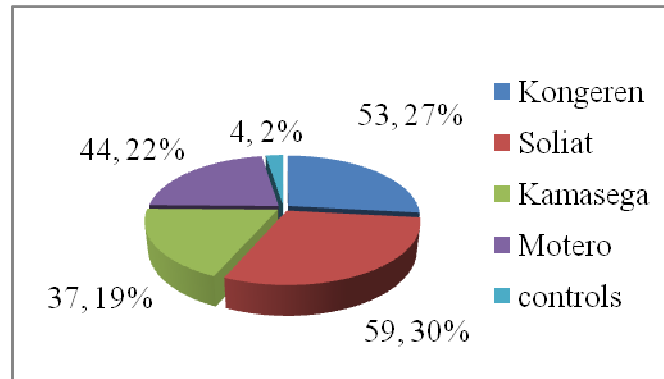


Figure 4. 1: Sample distribution in Soliat Location

4.2 Fungal Isolates from *Mursik*

Fungal contaminants grew on Yeast Potato Dextrose agar (YPDA) yielding mixed colonies of different fungal species (Plate 4.1). Out of 198 *mursik* samples 62.6% (124/198) were contaminated with *Geotrichum candidum* giving a higher prevalence. 33.8% (67/198) of the samples were contaminated with *Candida tropicalis*, 26.8% of the samples were contaminated with *Candida glabrata*, and 20.7% (41/198) colonized with *C. parapsilosis*. *C. albicans* contaminated only 11.1% of the *mursik* samples while 9.6% (19/198) of samples had *Rhodotorula* species. Both *C. kefir* and *C. krusei* contaminated 10.6% of samples and *Sacharomyces cerevisiae* contaminated only 6.6% (13/198) of samples (Table 4.1).

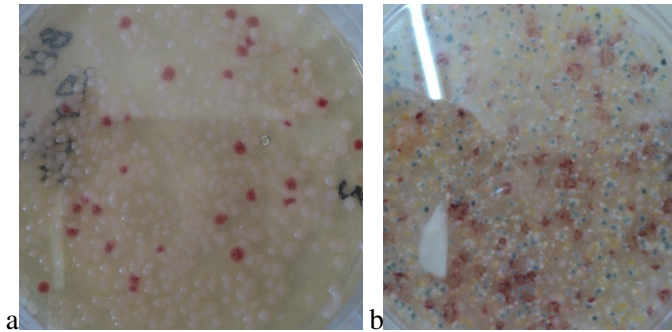


Plate 4. 1: Primary culture plates; with different colonies of yeast

Table 4. 1: Sample population contaminated by different yeast species

Fungal species	<i>f</i>	%	Samples not contaminated	% of samples not cont.	% of samples not cont.	Sample size
<i>G. candidum</i>	124	32.6	74	37.4	62.6	198
<i>C. parapsilosis</i>	41	10.8	157	79.3	20.7	198
<i>C. albicans</i>	22	5.8	176	88.9	11.1	198
<i>Rhodotorula spp.</i>	19	4.9	179	90.4	9.6	198
<i>C. tropicalis</i>	67	17.6	131	66.2	33.8	198
<i>C. kefyr</i>	21	5.5	177	89.4	10.6	198
<i>C. glabrata</i>	53	13.9	145	73.2	26.8	198
<i>S. cerevisiae</i>	13	3.4	185	93.4	6.6	198
<i>C. krusei</i>	21	5.5	177	89.4	10.6	198
Total	381	100				

Key: *f* – frequency of yeast species, %- percent, % – percent of yeast population, **cont.** – contaminated

A total of 381 yeast strains were isolated (Table 4.2). 32.6% (124/381) isolates were of *G. candidum*. *G. candidum* colonies showed white, dry powdery to cottony and grew rapidly. Microscopic morphology showed chains of hyaline, smooth single-celled, subglobose to cylindrical arthroconidia (Plate 4.2). 19/381 (5%) were colonies showing rapid growth, smooth, glistening soft and mucoid orange-yellow colour (Plate 4.2). Other colonies were white to cream, coloured, smooth, and glabrous yeast like in appearance. 13/381 (3.4%) on microscopy showed unicellular and elongate shaped multilateral budding cells and was confirmed to belong to *Sacharomyces cerevisiae* (6.6%). Other isolates fell under the genus

Candida where 22/381 (5.8%) had branched pseudohyphae and true hyphae and yielded green colonies on CHROMagar candida indicative of *Candida albicans* (Plate 4.3). 17% (67/381) gave blastoconidia and developed a blue colour on CHROMagar characteristic of *C. tropicalis*. 13.9% were *C. glabrata*, exhibiting cells, small in size with a light pinkish colonies on CHROMagar. *C. parapsilosis*, 10.8%, *C. kefyr* and *C. krusei* both at 5.5% showed pink, purple and whitish pink colours on CHROMagar respectively (Plate 4.3).

Table 4. 2: Yeast isolates and Colony colors of *Candida* species on CHROMagar *Candida* after 72 h incubation at 30°C

Fungal species	Number of Isolates (%)	Colors observed
<i>Geotricum candidum</i>	124 (32.6)	Blue with hyphae
<i>Candida parapsilosis</i>	41(10.7)	Pink
<i>Candida albicans</i>	22 (5.8)	Green
<i>Rhodotorula</i> species	19 (5.0)	Red
<i>Candida tropicalis</i>	67 (17.6)	Blue
<i>Candida kefyr</i>	21 (5.5)	purple
<i>Candida glabrata</i>	53 (13.9)	Light pink
<i>S. cerevisiae</i>	13 (3.4)	
<i>Candida krusei</i>	21 (5.5)	Whitish pink
Total	381 (100)	

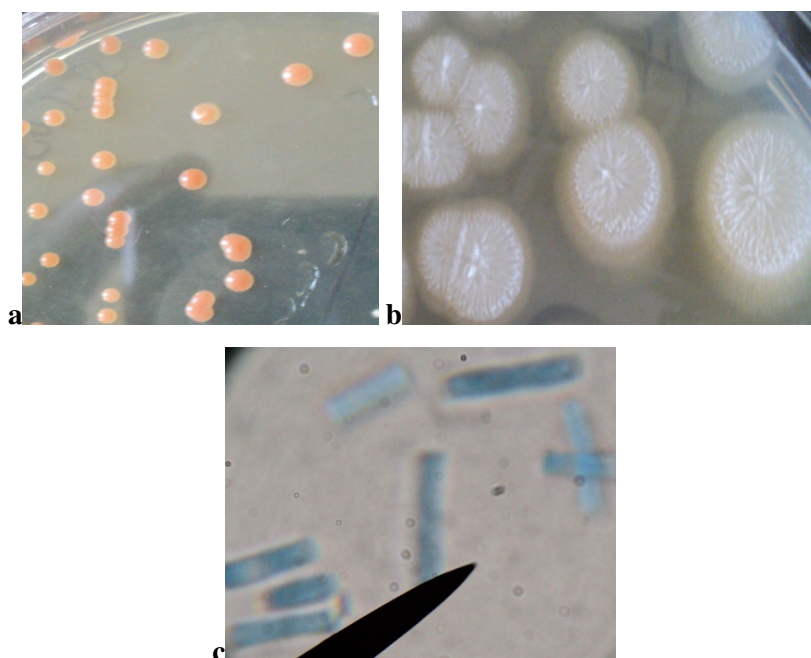


Plate 4. 2: Plate **a**; macroscopic appearance of *Rhodotorula* species showing: smooth, glistening soft and mucoid orange-yellow colour colony, and Plate **b**; *Geotricum candidum*, having a white, dry powdery to cottony. Plate **c**; microscopic characteristics of *G. candidum* showing hyaline, smooth, one-celled, subglobose to cylindrical arthroconidia.

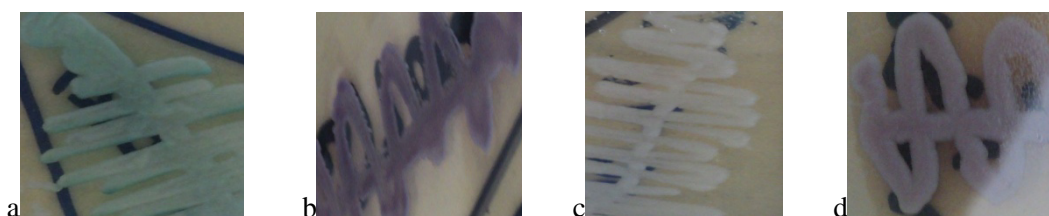


Plate 4. 3: Different *Candida* species on CHROMagar *Candida* (a) *Candida albicans*-green (b) *Candida parapsilosis*-pink (c) *Candida krusei*- whitish pink (d) *Candida glabrata*- light pink.

Analytical Profile Index (API 20 C AUX) tests (Table 4.3), confirmed the microorganisms to be *G. candidum* 124/381(32.6%), *C. tropicalis* 67/381(17.6%), *C. glabrata* 53/381(13.9%), *C. parapsilosis* 41/381(10.8%), *C. albicans* 22/381(5.8%), *C. kefyr* 21/381

(5.5%), *C. krusei* 21/381(5.5%), *Rhodotorula spp.* 19/381(5.0%) and *Sacharomyces cerevisiae* 13/381(3.4%).

Table 4. 3: Yeast fermentation of sugars; analytical profile index

Analytical Profile Index (API 20 C AUX) TESTS					
Fungal isolates	Glucose	Maltose	Galactose	Sucrose	Lactose
<i>G. candidum</i>	–	–	–	–	–
<i>S. cerevisiae</i>	+	+	+	+	–
<i>C. albicans</i>	+	+	+	–	–
<i>C. parapsilosis</i>	+	–	–	–	–
<i>C. kefir</i>	+	–	+	+	+
<i>C. glabrata</i>	+	–	–	–	–
<i>C. tropicalis</i>	+	+	+/_	+	–
<i>C. krusei</i>	+	—	–	–	–

Key; + fermentation, +/_ no clear fermentation and – no fermentation.

4.3 Agarose Gel Electrophoresis of Quantified Genomic DNA

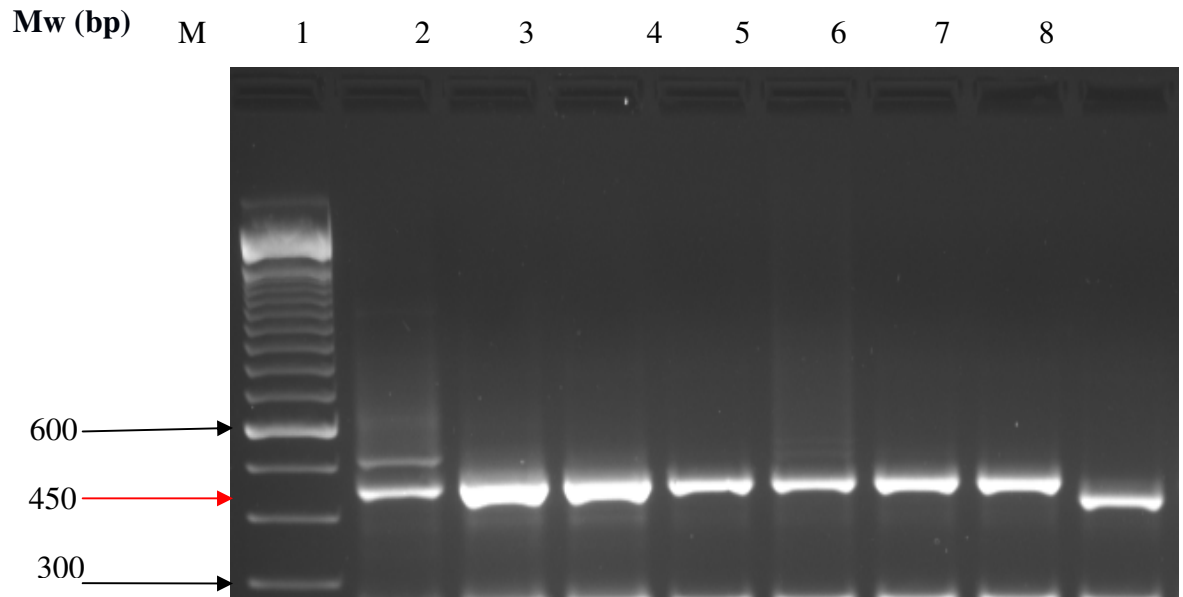


Plate 4. 4: PCR amplification profiles of beta tubulin. Arrow in red point to a 450 bp band. **M** represents a 1kb DNA ladder marker; Lane **1-8** represents the genomic DNA of fungi present in *mursik*.

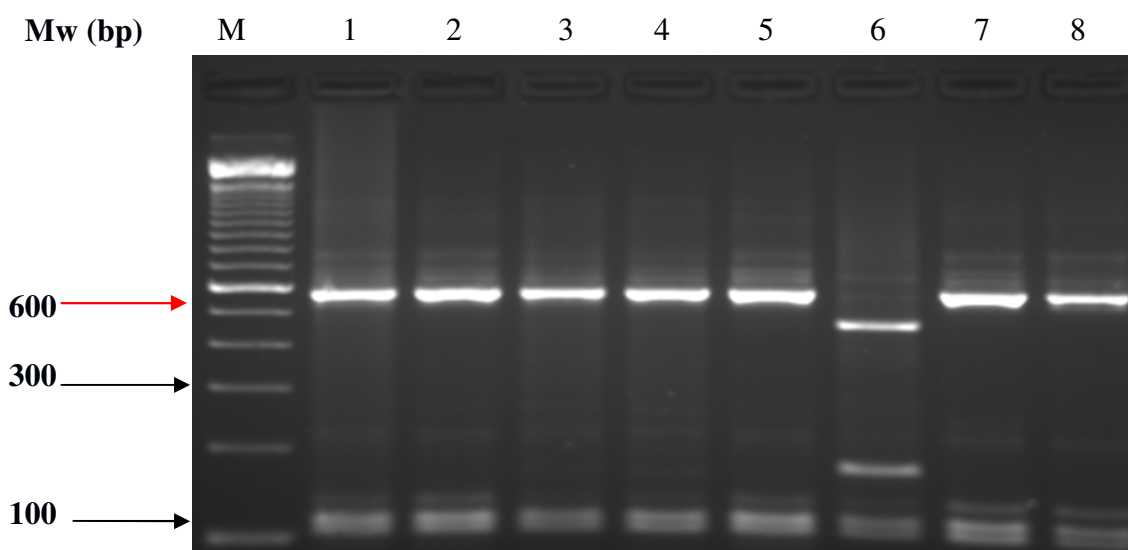


Plate 4. 5: PCR amplification profiles of rDNA ITS region. Arrow in Red points to a 600 bp band. **M** represents a 1kb DNA ladder marker; Lane **1-5, 7** and **8** represents the genomic rDNA of mycotoxigenic fungi present in *mursik*. Lane **6**, represents a different genomic DNA present in *mursik*.

4.4 Mycotoxins

Aflatoxins B₁, detected in 99.5% (196/198) of the samples with the range levels of 0-12ppb (Figure 4.2.). Fumonisin B₁ was detected in 3 (1.5%) of the samples had detectable quantities with the range levels of 0-0.77ppb (Table 4.4) and Deoxynivalenol toxins was detected on 1 (0.5%) sample only showing a quantifiable level of 0.25ppb (Table 4.5). The mean and range of the mycotoxins levels were calculated (Table 4.6).

Table 4. 4: Quantified levels of Fumonisin toxin in *mursik* collected in Soliat location in 2013.

Toxin level (ppb)	Frequency	% of total
0	195	98.5
0.33	1	0.5
0.39	1	0.5
0.77	1	0.5
	198	100

Table 4. 5: Quantified levels of DON toxin in *mursik* collected in Soliat location in 2013

Toxin level (ppb)	Frequency	% of total
0	197	99.5
0.25	1	0.5
Total	198	100

Table 4. 6: The mean and range of the mycotoxins levels in *mursik* collected in Soliat location in 2013.

	Aflatoxin(ppb)	Fumonisin(ppb)	Deoxynivalenol(ppb)
N	198	198	198
Mean	4.671	0.008	0.001
Range	12	0.77	0.25
Minimum	0	0	0
Maximum	12	0.77	0.25

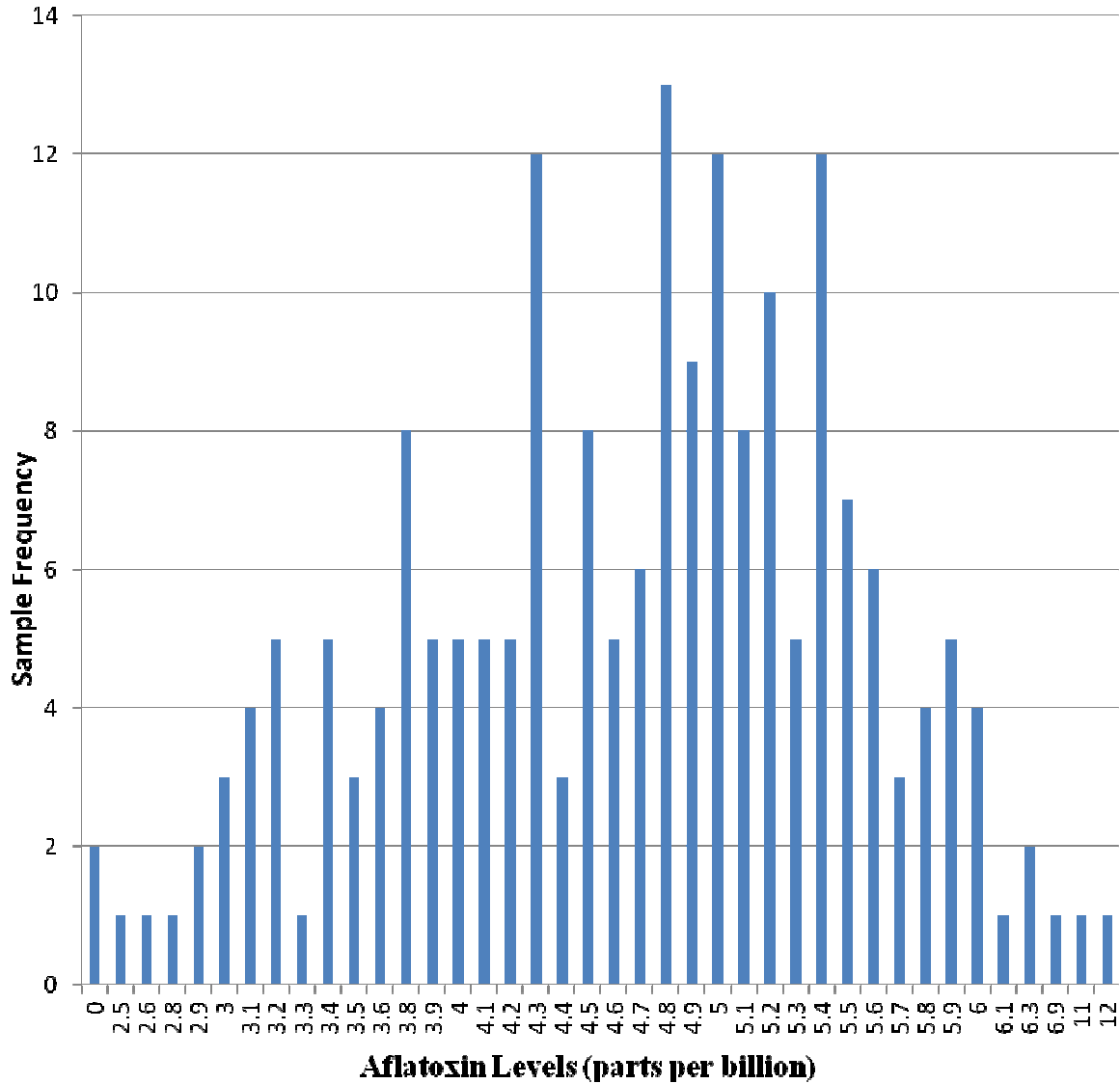


Figure 4. 2: Aflatoxin levels in *mursik* collected in Soliat location in 2013.

4.5 Other variables crucial in *mursik* processing

From the questionnaire results, all participants were milk fermenters. 85.3% of the farmers obtained milk for fermentation from their own cows, 14.7% bought from other farmers. Participants reportedly milked their cows in the open field. After milking, 90% of the participants did not cover the milk containers. Among the respondents, 88% did not boil milk before fermentation. All people used calabashes to ferment their milk, and left it for

duration of 3-4 days, with 82.2% and 17.8% keeping it for 3 and 4 days respectively. Furthermore, none of the respondents have ever used starter cultures. Charcoal like material (wo'sek), obtained from *Senna didymobotrya* plant, and was used to treat calabashes, before pouring fresh milk, then left to ferment. Calabashes containing milk underwent fermentation process in living rooms (among 74% of respondents) as well as maize stores (74% of the participant). Use of hot water to clean calabashes was reported by 80% of participants, with 20% utilizing cold water. The clean calabashes were subsequently used in further fermentation. As the capacity of calabashes varied, coupled with the fact that cattle kept produced small quantities of milk, addition of more fresh milk into the calabash containing already fermenting milk was reportedly done after every 1 day by all farmers until it filled up, before leaving to ferment. Commercial feeds were not used by participants. In addition to grazing their cattle freely in the field, they supplemented it with nappier grass.

CHAPTER FIVE

5 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Fungal contaminants

The results obtained from the microbial analysis of *Mursik* in this study show that product was contaminated with microorganisms of public health concern. The fungal isolates constituted mainly the yeasts. The present study isolated 381 strains of the following fungi, *Geotrichum candidum*, *Candida tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. albicans*, *C. kefyi*, *C. krusei*, *Rhodotorula spp* and *Sacharomyces cerevisiae*. Microbiological analyses provide useful information which shows the conditions in which the milk was obtained, processed and stored (Pietrowski *et al.*, 2008).

G. candidum has been known to be the most important species in food and identified as the dairy mold which contributes to flavour and aroma development in cheese and is also responsible for the characteristic taste and aroma of *gari*, a fermented cassava product (Jay, 1992). It is thus expected that *G. candidum* plays a functional role in the development of the taste and flavour of *mursik*. However, geotrichosis is an infrequent opportunistic mycosis whose main etiologic agent is *Geotrichum candidum* though several studies have proven that it is a commensal in humans and part of the normal flora of the skin, mouth and gastrointestinal tract (Pottier *et al.*, 2008). Many reported clinical cases have occurred in immunosuppressed patients or immunosuppressive disorders, and has been known to affect the respiratory tract (bronchi and lungs); superficial skin infections and lately invasive infections have also been reported (Girmenia *et al.*, 2005) and oral geotrichosis has an association with diabetes mellitus (Hattori *et al.*, 2007). *G. candidum* with its positive contribution in fermentation, it poses treat to consumers especially persons whose immune systems are compromised.

Candida tropicalis, has been isolated from *sobia*, a fermented beverage in the western province of Saudi Arabia (Gassem, 2002), and some traditional fermented milk products in Zimbabwe (Gadaga *et al.*, 2000). *C. tropicalis* has been identified as an important yeast pathogen of humans (Ahearn, 1998) and its presence in the traditionally fermented milk indicates that these fermented foods could be a source of pathogenic microorganisms.

C. glabrata, *C. albicans* and *C. parapsilosis* were isolated by Gogoi *et al.*, (2014) in mark raw milk. *C. albicans* causes candidiasis in humans and has been associated with infections, as well as colonization, in both immunocompromised and immunocompetent patients. Due to rampant cases of HIV/AIDS, some other *Candida* species other than *C. albicans* have also emerged as significant opportunistic fungal pathogens (Richardson & Warnock, 2003). Examples are *C. glabrata*, *C. parapsilosis*, *C. kefyr* and *C. krusei* which have been responsible for increasing the proportion of cases of fungemia and other complex cases of candidiasis (Colombo *et al.* 2006; Lewis *et al.* 2009; Miceli *et al.* 2011). In terms of virulence and pathogenicity, these species behave with equal or greater virulence compared with *C. albicans* (Abbas *et al.* 2000).

Milk fermentation has been known to result in the production of ethanol resulting in acetaldehyde (ACH) which is a carcinogen especially responsible mostly for esophageal cancer (Mikko *et al.*, 2012). According to Salaspuro, (2009), acetaldehyde (ACH), resulting from ethanol or tobacco, is responsible for the pathogenesis of cancer. It has been concluded that ACH present in alcoholic beverages, is a congenitor or formed endogenously from ethanol and is a Group1 carcinogen in humans (Secretan *et al.*, 2009). Esophageal squamous cell cancer (OSCC) is unusually frequent in southern and eastern Africa, including Western Kenya, where it is the most common malignancy in both men and women. Of special interest is that occurrence of OSCC is in young people of less than 30 years of age in the area, especially among members of the Kalenjin tribe (Lodenyo *et al.*, 2005). A study done by Mikko *et al.*, (2012), provided evidence that high levels of ACH and alcohol are present in mursik having upto 3.8% w/v ethanol and mutagenic concentrations of ACH. This has been postulated to be responsible for the high prevalence of esophageal cancer cases occurring in patients 30 years of age or younger amongst the

Kalenjins community. *Candida albicans* is known to produce significant amounts of ACH during fermentation. The presence of *Candida albicans* in *Mursik* is therefore a significant risk factor for ACH exposure. Other yeasts that have been known to produce ACH are *Candida kefir* in conjunction with *Lactobacilli* bacteria.

Candida kefir, are some of the most predominant and important yeast species in milk (Fleet, 1990). *C. kefir* is able to assimilate lactose, and therefore has a potential for growth in milk (Gadaga *et al.*, 2000). *C. kefir* has been known to produce negligible amounts of lactic acid and inclusion of the yeast into mixed cultures did not seem to affect production of lactic acid by the LAB (Gadaga *et al.*, 2001).

C. krusei as has been known to be used together with lactic acid bacteria as starter cultures to uphold the activity and enhance the long life of the lactic acid bacteria, even though this kind of association are not confirmed but *C. krusei* cannot metabolise lactose, although is able to utilise the breakdown products made available by lactose metabolism by *Lactobacilli*, which is a strong proof for the association (Frazier & Westhoff, 2001; Mikko *et al.*, 2012). This showed that symbiotic association between *C. krusei* and the lactic acid bacteria is involved in *mursik* production. *C. krusei* is also drawn in the fermentation of cacao beans, playing a vital role in development of the desirable chocolate flavour of roasted beans. Flavor improvement is attributed to the proteolytic ability of the yeasts (Jay, 1992). Thus *C. krusei* may too play a role in flavour improvement in *mursik*.

Rhodotorula species are the mainly frequent species in foods especially *R. mucilaginosa* and *R. glutinis*. They produce pink or red pigments and cause discolouration of foods such as fresh poultry, fish, beef and sauerkraut (Jay, 1992; Frazier & Westhoff, 2001) giving bitter taste to the products (Muhamad *et al.*, 2006). Pitt and Hocking (1999), reported them to cause spoilage in dairy products such as yoghurt, butter, cream and cheese. Consequently the presence of *Rhodotorula* in *mursik* is unwanted as its existence in milk products results to spoilage.

Saccharomyces cerevisiae has been known to be of beneficial role in cheeses and fermented milk for example *kefir* and *koumiss*. *S. cerevisiae* 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). Zimbabwean traditionally fermented milk *S. cerevisiae* enhanced growth of *L. lactis* subsp. *Lactis* and additionally stimulated growth of *Lb. paracasei* in UHT milk (Gadaga *et al.*, 2001). These therefore indicate that presence of *S. cerevisiae* in *mursik* contributes in the improvement of flavour; aroma and enhancement of milk lactobacilli as it inhibits the growth of harmful organisms improving on the value of the milk product. *S. cerevisiae* has been considered as a well-established cause of nosocomially acquired yeast infection. Its fungemia is unknown but considered a risk factor for nosocomial bloodstream infection in patients with predisposing underlying conditions (Lherm *et al.*, 2002). The most consistent risk factor for *S. cerevisiae* fungemia is the use of probiotics. *S. cerevisiae* can cause a wide variety of clinical syndromes, like liver abscess and esophagitis (Aucott *et al.*, 1990; Konecny *et al.*, 1999). This implies that presence of *S. cerevisiae* in *mursik* and its use in other fermented foods raises clinical concerns to the end users of the products.

Yeast species can produce several extracellular enzymes, mainly proteinases, phospholipases and lysophospholipases. These hydrolytic enzymes can modify the membrane components, causing a malfunctioning. Pathogenic *Candida albicans* strains have been known to produce toxins though toxin production appears to be a strain characteristic and not a property of all pathogenic strains. These toxins could be in two groups: high-molecular-weight-toxins (candidotoxin) and low molecular-weight-toxins. Both toxins affect mechanisms of humoral immunological response affecting the interaction between macrophages and fungi. Acid phosphatase with pH optima at 3-6 and 5.6, a peptidase with optimum pH 6.6 and β -glucosidase have been isolated from both blastospore and mycelial forms of *C. albicans* and have been indicted as the potential agents of pathogenicity because of their proteolytic activities (Chattaway *et al.*, 1971).

During fungal investigations, moulds were not isolated. The use of charcoal (wo'sek) from *Senna didymobotrya* could have inhibitory activity on moulds. Reports by Korir *et al.*, (2012) indicated that the extracts from *Senna didymobotrya* have inhibitory properties against filamentous fungi. The use of the charcoal from the plant could also be to act as a detoxifying agent to binding toxins and carcinogens in the fermenting milk (Mikko *et al.*,

2012). Therefore the absence of moulds or mycotoxin producing fungi from the *mursik* could be due to the antifungal properties of the plant.

However, DNA isolated from 198 samples of *mursik* was used as template and PCR was carried out with two combinations of primer pairs: ITS5/ITS4 that amplifies the 18S-28S rDNA region, and Bt2a/Bt2b that amplify the 400- 500bp region. When primer pair Bt2a/Bt2b was used, a unique amplicon of approximately 450 bp in size was obtained for all tested samples. No significant differences in amplicon size were noted among the various samples tested (Plate 4.4). With the primer pair ITS5/ITS4, an amplicon of approximately 600 bp was obtained for all samples apart from 1 sample which gave an amplicon of approximately 580 bp a subject of further analysis through sequencing (Plate 4.5). The results suggest contamination of *mursik* with mycotoxigenic fungi which is in accordance with the data from literature (Mirhendi *et al.*, 2007). Similar amplicons, as size, were obtained for *A. ochraceus* strains (Aguirre *et al.*, 2004).

Moulds of the genus *Aspergillus*, *Penicillium* and *Fumonisin* were among the contaminants of *mursik*. *Aspergillus niger*, *Penicillium chrysogenum*, *Rhizopus* spp, *Fusarium moniliforme*, *Trichoderma reesii*, and *Aspergillus fumigatus* are among the contaminants of traditionally fermented milk (Uzeh *et al.*, 2006). Though, they have been associated to fermentations of milk products. Several fungal starter cultures commonly used in Asia have been used in fermentations in other places as fungi can add fiber, vitamins and proteins to fermented foods (Nout, 1994).

Aspergillus species for instance *Aspergillus oryzae* and *A. sojae* are used in the production of miso and soya sauce fermentations. *Aspergillus oryzae* and *A. Niger* are also used for production of sake and awamori liquors, respectively (Nout, 1994). *Aspergillus acidus* is used for fermenting Puerh tea. The presence of the *Aspergillus* species in the traditional fermented milk; *mursik* could be responsible for its fermentation. On the other hand, some members of the genus *Aspergillus* are contaminants of milk product and contribute to its spoilage. *Aspergillus* and *Rhizopus* species were the moulds isolated as the primary contaminants in yogurt produced in Nigeria (Ifeanyi *et al.*, 2013). *Aspergilli* species have been reported as spoilage organisms during storage of a wide range of foods where they

may produce mycotoxins. Mycotoxin produced by *Aspergillus* is known as aflatoxin which has been among the toxins contaminating milk (Kang'ethe & Lang'a, 2009).

Moulds of the genus *Penicillium* is most frequently occurring on cheeses and has been used in Europe to ripened foods, primarily cheeses and meats, usually using a *Penicillium* species. This indicated that presence of penicilium could contribute to the fermentation. However, *Penicillium* species are reported to produce the widest range of mycotoxins. Among them are ochratoxin A; patulin; citrinin; citreoviridin and griseofulvin (Leistner, 1990). Mycotoxins produced by *Aspergillus* and *Penicillium* have been known to cause mycotoxicoses. These toxins can damage liver, causing cirrhosis and can also induce tumor (Sell *et al.*, 1991). Hence the consumption of the fermented milk in area of study contaminated with these moulds are dangerous to health since it is a potential sources of mycotoxicosis.

Fusarium species has been used in the fermentation of cheese for example *Fusarium domesticum* has been used for cheese fermentations (cheese smear). *Fusarium solani* was isolated from a Vacherin cheese, and is used extensively for mycoprotein production in Europe (Thrane, 2007). Thus, its presence could be contributing to some extent the fermentation of milk. However, *Fusarium* species have been known to produce fumonisin and trichothecene mycotoxins as their secondary metabolites (Thrane, 2007; Marasas, 2001) and it will contribute to the contamination of milk and milk leading to the risk of mycosis to the consumers.

Data from this study is similar to the work reported by Abd El- Aziz *et al.*, (2011) who showed that fungi of the genus *Aspergillus* spp., *Penicillium* spp., *Rhizopus* spp and *Candida* spp. were present in fermented camel milk. The existence of yeasts and moulds in a range of spontaneously fermented milk products has been reported by other by researchers (Gadaga *et al.*, 2000; Abdelgadir *et al.*, 2001; Lore *et al.*, 2005; Kabede *et al.*, 2007; Zhang *et al.*, 2008; Rahman *et al.*, 2009). Presence of yeasts in naturally fermented milk products may be potential cause of spoilage but contribute to development of texture and flavor (Narvhus & Gadaga, 2003).

From the study no starter cultures are used; fermentation is initiated by the natural microorganisms of the milk. Milk is left to ferment naturally at room temperatures (Eyassu *et al.*, 2012). Before fermenting, a remarkable number of respondents did not boil the milk. Moreover, as reported by Chelule *et al.*, (2010) and Nakavuma *et al.*, (2011), calabashes were the preferred containers for the fermentation process, which took between 3-4 days. Prior to pouring fresh milk into them, calabashes were treated and seasoned with charcoal like material (wo'sek), obtained from *Senna didymobotrya* plant (Mureithi *et al.*, 2000).

Equal number of people fermented their milk in living rooms as well as maize stores. These conditions leaves the milk and the containers contaminated by fungal spores since they can be found in the atmosphere and from those fungi for example, *Aspergillus*, *Fusarium*, and *Penicillium* that attack maize in the stores (Mboya *et al.*, 2011).

As the capacity of calabashes varied, coupled with the fact that cattle kept produced small quantities of milk, addition of more fresh milk into the calabash containing milk at various stages of fermentation was done after every 1 day. Upon consumption of the fermented milk, majority of the respondents cleaned their calabashes using hot water which was also reported by Eyassu *et al.*, (2012). Nevertheless, a small percentage employed cold water. The clean calabashes were subsequently used for further fermentation.

In the present study, participants milked their cows in the open field. Thereafter, a significant percentage of them did not cover the milk containers during transportation to the living houses leaving milk vulnerable for microbial contamination. Other studies have shown that many microorganisms are transmitted during milking. Fungi are ubiquitous in the environmental and present in the air, water, feaces, and in the cow shade. These include: moulds, yeasts, algae among others. These microorganisms include: *Cryptococcuss spp.*, *Rhodotorula spp.*, *Aspergillus spp.*, *Pichia*, *C. krusei*, *C.tropicalis*, *C. albicans*, *Trichosporon spp.* (Ludmilla *et al.*, 2011). It is certain that unhygienic milking procedures and equipment used for milking, storing or distributing milk may have been an important source of the isolated microorganisms. This confirms past studies done by Bryan, 1983. He also suggested that, milk should be stored under conditions which prevent mould

growth and strict hygienic measures and regulations should be imposed during processing, packaging and transportation.

Other studies shows that different yeast species were predominant in different fermented milk products for example, *Sameel* which is a traditionally fermented milk in Saudi Arabia, yeast isolated include; *Candida spp.*, *Cryptococcus laurenti*, *Candida kefir*, and *Sacharomyces cerevisiae* (Bozena *et al.*, 2011). According to Gadaga *et al* (2000), *C. lusitaniae*, *C. colliculosa* and *S. cerevisiae* were the isolated strains from Zimbabwean traditional fermented milk. *Debaryomyces hansenii* was the predominant strain in Sardinian ewe's dairy products (Cosentino *et al.*, 2001). Abdelgadir *et al.*, (2001) in Sudan and Shuangquan *et al.*, (2006) in Mongolia, found that the predominant yeast strains in the fermented milk were *S. cerevisiae* and *C. kefir*. *C. krusei* while *C. kefir* has been found to be the most dominant yeast species in Ugandan traditionally fermented milk; 'Kwerionik' (Nakavuma *et al.*, 2011). In this study, *C. krusei* and *C. kefir* were the least isolated species which was also echoed by Al-Otaibi, (2012).

Bozena *et al* (2011), isolated yeasts from milk of the following species; *C. parapsilosis* and *C. krusei* being predominant and *C. tropicalis* and *C. albicans*. Other yeasts of the genus, *Geotricum*, *Sacharomyces* and *Rhodotorula* as the least isolated but in the present study, the predominant yeast was *Geotricum candidum*. Others isolated by Bozena *et al.*, (2011), were from the genus *Cryptococcus* and *Trichosporon*. Therefore, use of contaminated milk by yeast of the genus, *Candida spp.*, or by toxins produced by them may be harmful to human health. Given that the product does not go through any pasteurization, consumers, particularly immunocompromised individuals are at risk of acquiring opportunistic mycoses (Ludmilla *et al.*, 2011).

Fungi of the genus *Candida*: *C. krusei*, *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. lusitaniae*, *C. holmii*, *C.lambica* have been identified in the crude milk directly derived 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). The microflora involved in the production of *suusac*; Kenyan traditionally fermented milk produced by Somalis, are yeasts

namely; *C. krusei*, and *Rhodotorula muciliginosa* (Lore *et al.*, 2005). *C. albicans*, though infrequently isolated from milk, grow well in the milk at all temperatures. Thus bringing a lot of concern since this organism is known to be an opportunistic pathogen in humans (Kabede *et al.*, 2007).

Finding from the present study showed that moulds of the genus *Aspergillus*, *Penicillium* and *Fusarium* were indeed responsible for the contamination of milk. Indeed, the three genera have been known to be the main mycotoxin producing moulds (Lanyasunya *et al.*, 2005). Furthermore, *Aspergillus spp.*, *Alternaria spp.*, *Fusarium spp.*, *Neurospora spp.* are the most common fungi species found in milk products (Saadia, 2010). This result confirms what has been reported by other researchers. According to Uzeh *et al.*, (2006), moulds of the following genus; *Aspergillus*, *Penicillium*, *Rhizopus*, *Fusarium*, and *Trichoderma* species have been isolated. Known spore formers, of mycotoxigenic fungi have been isolated from *nono* and *wara*; the traditionally fermented milk product.

The species of the genus *Aspergillus*, *Penicillium* and *Fusarium* were isolated by Pešić-Mikulec *et al.*, (2005) in raw milk. Abd El- Aziz *et al.*, (2011) isolated and genetically identify moulds from camel milk of the genus, *Aspergillus*, *Penicillium*, *Mucor* and *Rhizopus* and revealed that *Penicillium spp.* was the predominant mould isolates. *Aspergillus niger* was the most common mould isolates as reported by Chhabra *et al.*, (1998). Marcos *et al.*, (1990) explained that *A. fumigatus* was the most frequent mould and (Prabhakar *et al.*, 1989) affirmed that *mucor spp* was the regular mould contaminat on cow milk. They are major spoilage organisms of carbohydrate foods (Rhodes & Fletcher, 1966). However, their growth can result in the production and accumulation of mycotoxins which are of public health and economic importance (Uzeh *et al.*, 2006).

Other documented milk contaminants includes: *Aspergillus glaucus*, *A. niger*, *Alternaria spp.*, *Cheotomium candidum*, *Cladosporium herbarum*, *Fusarium spp.*, *Monilia spp.*, *Mucor rouxii*, *Neurospora spp.*, *Penicillium expansum*, *Penicillium spp.*, *Rhizopus nigricans*, *Sporotrichum carinis* and *Thamnidium elegans*. Fungi of the genus, *Fusarium*, *Aspergillus*, *Penicillium*, *Cladosporium*, *Geotrichum* and *Cladosporium species* have been detected in milk during different seasons in different geographical locations (Pešić-Mikulec

et al., 2000). Findings from the current study, therefore confirms these past studies. Uzeh *et al.*, (2006), suggested that, it is important to prevent mould growth to avoid toxin production by preventing the natural contamination of raw materials and the microbiological standards be put in place for locally processed dairy products.

Commercial feeds which normally are from cereals were not used by participants (Lanyasunya *et al.*, 2005). In addition to grazing their cattle freely in the field, they supplemented it with nappier grass. Cereals, used as cattle feed, forms a very good substrates for fungal growth such as: *Aspergillus*, *Penicillium* and *Fusarium* species and susceptible to mycotoxin contamination (Sultana *et al.*, 2009). Further, fungi responsible for these toxin productions can colonize milk, and thus producing significant amounts of toxins under poor handling practices (Lanyasunya *et al.*, 2005).

In the current study, majority of farmers, fermentation process took place in cereal stores. Importantly, the calabashes were opened daily, for further addition of fresh milk, hence increasing the likely hood of contamination with *Aspergillus*, *Penicillium* and *Fusarium* species, as reported in the study.

5.1.2 Fungal mycotoxin genes

With the molecular detection of mycotoxigenic fungi in *mursik*, it is a clear indication that species of *Aspergillus*, *Penicillium* and *Fusarium* are present and carry the genes responisple for toxin synthesis. Early studies by Schindler *et al.*, (1967) showed that aflatoxin was produced maximally at 24°C and not at all at temperatures lower than 18°C or higher than 35°C. Obrian *et al.*, (2007), indicated that, aflatoxin production was at the highest at 28°C and decreasing amounts were produced as temperature increased from 34-37°C. However, Diener and Davis, (1967) reported aflatoxin production in peanuts at 40°C by *A. flavus*. Mayne *et al.*, (1967) suggested that the effect of temperature is more dependent on substrate than on strain. Feng and Leonard, (1998) compared *A. parasiticus* to *A. nidulans* under varying culture conditions. They detected aflatoxin at 27°C and lesser amounts at 33°C but were unable to detect aflatoxin in *A. parasiticus* at 37°C. In contrast they found that *A. nidulans* produced sterigmatocystin at similar levels at all three temperatures. While the cardinal range for aflatoxin production differs among strains and

culture conditions, most research shows temperatures between 24-30°C favor aflatoxin biosynthesis (Obrian *et al.*, 2007). *Fusarium* species has shown to grow well at 26 to 28°C; fumonisin and DON toxins are detected at its maximum (Reid *et al.*, 1999).

Both transcriptional and posttranscriptional regulation mechanisms control aflatoxin gene transcription (Yu *et al.*, 2004a). Northern analysis has been used to show the aflatoxin polyketide synthase gene of *A. parasiticus* is expressed at 27°C, another path way gene, *omtA* also was shown to be transcribed in *A. parasiticus* at 29°C and at 28°C, but not at 37°C (Feng & Leonard, 1995; Liu & Chu, 1998; Obrian *et al.*, 2007). The norsolorinic acid reductase, O-methyl transferase B and aflatoxin polyketide synthase were highly expressed at temperature of 28°C (Obrian *et al.*, 2007). The area of study has the temperatures of between 18°C-37°C (Kang'ethe & Lang'a, 2009). Participants ferment their milk using guards at room temperature, thus favouring the growth of mycotoxigenic fungi. Thus, the area temperature and their (participants) fermenting temperature promotes expression of aflatoxin, fumonisin and DON synthesis genes leading to mycotoxin contamination.

Aspergillus and *Fusarium* species has been shown to grow well on high water activity of 0.8-0.90_{a_w}. The *nor-1* gene responsible in the process of Aflatoxin synthesis is highly expressed at 0.90_{a_w} with less at 0.95_{a_w} and 0.85_{a_w} (Abdel-Hadi *et al.*, 2010). Fermented milk (*mursik*) has water activity of 0.90_{a_w}, thus promoting the contamination of mycotoxigenic fungi especially of *Aspergillus*, *Fusarium* and favor the expression of *nor-1*, *FUM1* and *tri* genes in the fungi leading to mycotoxin contamination (Chelule *et al.*, 2010). *Mursik* pH has been known to be a low at 5, this brings *mursik* to be contaminated as the fungal contaminants grows well at pH 5 (Nakavuma *et al.*, 2011).

5.1.3 Aflatoxins, Fumonisin and Deoxynivalenol (DON) or Vomitoxin

In the current study, 99.5% of the samples were contaminated with Aflatoxins. Of these samples, the average level of contamination was 4.67 ppb levels exceeding 0.05ppb as per EU, FAO/WHO and Food and Drug Administration (FDA) permissible levels for milk (Kang'ethe & Lang'a, 2009). Concerning Fumonisin the current study showed a remarkably low contamination of samples (1.5%) and the mean concentration of contamination was 0.008 ppb. 0.5% of the samples were contaminated with

Deoxynivalenol toxins, with an average contamination of 0.001 ppb lower than the recommended levels of 0.05 ppb of EU, FDA and FAO/WHO.

The disease caused by ingestion of aflatoxins in contaminated foods is called aflatoxicosis. Acute aflatoxicosis occurs when aflatoxins are consumed at moderate to high levels. *A. flavus* is the second leading cause of aflatoxins, which commonly affects maize, causes illness and even death when consumed in large quantities. Low-level, chronic exposure is carcinogenic and has been linked to growth in children (Strosnider *et al.*, 2006).

The levels of contamination of fermented milk aflatoxins reported in this study are similar to those reported in Kenya by Kang'ethe and Lang'a, (2009) where samples, 99% were positive for aflatoxins. Reports of contamination of milk in various parts of the world have been reported by De Sylos *et al* (1996), in Brazil, Rousi *et al* (2002) in Greece and Diaz *et al* (2006) in Colombia. However, in this study a higher proportion of samples exceeding the FAO/WHO limit of 0.05ppb are reported. Higher proportions have been reported in India where 99% of the contaminated raw milk, milk based cereal weaning formula and infant formula exceeded the 0.05ppb (Dwivedi *et al.*, 2004).

Major outbreaks of acute aflatoxicosis from contaminated food in humans were reported in developing countries (Centers for Disease Control and Prevention, 2004; Lewis *et al.*, 2005). For example, in western India in 1974, 108 persons died among 397 people affected with aflatoxin poisoning in more than 150 villages (Krishnamachari *et al.*, 1975). A more recent incident of aflatoxin poisoning occurred in Kenya in July 2004 leading to the death of 125 people among 317 reported with illness due to consumption of aflatoxin contaminated maize (corn) (Centers for Disease Control and Prevention, 2004; Lewis *et al.*, 2005). Acute toxicosis is not the only concern. World health authorities warn that low doses and long term dietary exposure to aflatoxins is also a major risk as chronic exposure can lead to hepatocellular carcinoma (Fung & Clark, 2004).

Depending on the level and duration of exposure, aflatoxins possess both hepatotoxic and carcinogenic properties. Symptoms in humans include vomiting, abdominal pain, alteration in digestion, limb and pulmonary edema, convulsions, rapid progressive jaundice, swollen liver, high fever, coma, and death. The predominant damage is to the liver (Lewis *et al.*,

2005), but acute damage to the kidneys and heart have been reported (Richard & Payne, 2003). In liver aflatoxins irreversibly bind to protein and DNA to form adducts such as aflatoxin B1-lysine in albumin and a guanyl-N7 adduct in DNA (Skipper & Tannenbaum, 1990). Disruption of the proteins and DNA bases in hepatocytes causes the toxicity (Azziz-Baumgartner *et al.*, 2005).

The gene mutation of p53 tumour suppressor gene leads to initiation of hepatocarcinoma formation (Coursaget *et al.*, 1993). Human hepatocarcinomas are also associated with hepatitis B virus (HBV) and C virus (HCV) infections (Wild *et al.*, 1992). Together with aflatoxins these viruses significantly increased the risk of hepatoma in hepatitis patients (Arsura & Cavin, 2005). In developing countries, many children are exposed to aflatoxin before birth, while nursing and after weaning (Turner *et al.*, 2007; Polychronaki *et al.*, 2007; Gong *et al.*, 2004). An association of hepatocellular carcinoma and dietary exposure with aflatoxins has been established from patients living in high-risk areas of China, Kenya, Mozambique, Phillipines, Swaziland, Thailand, Transkei of South Africa (Lewis *et al.*, 2005). The present study indicates that milk are contaminated with mycotoxins though there is less work have been done to uncover the real extent to which milk and milk products are contaminated. This indicates that tcontamination of milk and its products with this toxin are of great concern to the public health.

Fumonisin toxin was detected in 1.5 % (3) of the samples and it was below the acceptable limits. Same results were reported by Maragos and Richard (1994) who analyzed 155 milk samples collected in Wisconsin during a period when feeds were reported to be severely affected by mold. Only one of the 165 milk samples tested positive for fumonisin, which was determined to be below the set acceptable levels. Fumonisin carryover from feed to milk has been indicated to be negligible (Richard *et al.*, 1996; Scott *et al.*, 1994). Prelusky *et al.* (1996) reported studies where dairy cattle were administered fumonisin orally and no fumonisin or its metabolites were detected in milk. This and results from this study suggests that fumonisin can occur in milk, but at very low levels.

Fumonisins are mainly produced by *Fusarium* species, for example, *F. verticillioides*. Fumonisin have been classified into three fumonisins B1 (FB1), B2 (FB2) and B3 (FB3)

which occur normally in abundant levels (Sreenivasa, 2012). Fumonisin in humans has been related with neural tube defects in people that rely on maize as a staple food, but most prominently it has been indicated as a potential cause of oesophageal cancer in humans (Missmer *et al.*, 2006; Marasas, 2001). In certain regions around the world including Bomet County in Kenya, where high daily intake of maize and maize-derived commodities occurs, an associations between either mouldy maize, *F. verticillioides* or fumonisin and the incidence of oesophageal cancer have been reported (Marasas, 2001; Doko & Visconti, 1994).

In dietary exposure to FBs has been linked to high incidences of oesophageal cancer observed in Transkei in South Africa, North East Italy China and Iran (Isaacson, 2005; Francheschi *et al.*, 1990; Sun *et al.*, 2007; Shephard *et al.*, 2000). In 2005 and 2006, fumonisin exposure was seen in Tanzania and was attributed to the dry seasons in the two years (Kimanya *et al.*, 2009). It was isolated at high levels in corn meal and corn grits, including seven samples from a supermarket in Charleston, South Carolina, the city with the highest incidence of esophageal cancer among African Americans in the United States (Sydenham *et al.*, 1991).

The toxic concentrations of fumonisin differ much depending on the animal species (Rheeder *et al.*, 2002). Absorption of about 5 - 10 ppb fumonisin in feed induces neurotoxic effects in horses (Marasas *et al.*, 1988). In pigs the ingestion of 4 - 16 ppb body weight may result in liver cirrhosis and more than 16 ppb body weight result in pulmonary edema (Marasas, 1995). Chickens can withstand higher concentrations of fumonisin in feed, up to 75 mg/kg and cattle seem not to be affected by high fumonisin concentrations (Gelderblom *et al.*, 1991). With the low levels of the fumonisins detected in *mursik*, it is an indication the the milk product is safe from the contamination but it can arise due to the poor handling of the product processing.

Deoxynivalenol (DON) is a secondary metabolite produced by a *Fusarium* species. It is one of the mycotoxins most commonly detected in feeds. DON is also called vomitoxin because it was first associated with vomiting in swine. Studies have shown DON to be a primary mycotoxin associated with swine disorders including feed refusals, diarrhea,

reproductive failure, and deaths. Dairy cattle consuming feeds contaminated primarily with DON have led to reduction in milk production. Research has shown that DON causes polyribosomal breakdown in mammalian cell lines (Whitlow *et al.*, 2005).

DON contamination of milk in this study was detected in one sample which was below the EU, FDA, and FAO/WHO acceptable levels and other samples were below the detectable limits. This results are in agreement with results of Seeling *et al.*, (2006) where they established that DON in milk was below the detectable levels. *Fusarium* species has been commonly found contaminating or colonizing cereals and their products, stuffs constituting an important part of human food and animal feed. Highly contaminated crops are frequently directed to animal feed (Bennett & Klich, 2003). DON transfer in animal and the possible presence of toxic residues in animal products for example milk, meat remain unclear (Cavret & Lecoer, 2006). It has been presented that, DON toxins are detected in milk from animals fed with DON contaminated feeds and that the detection limits increased with the increased levels of contamination of feeds but it did not correlate with the amounts fed to the animals; the toxins detected were below the levels fed to the cows (Seeling, 2006).

Results from this study also confirm Seeling *et al.*, (2006); Cavret and Lecoer, (2006). The participants, used *Senna didymobotrya* plant product to pretreat their milk before fermented which has been known to be fungicidal (Korir *et al.*, 2012) and must have inhibit further establishment of the *Fusarium* fungus in milk thus no metabolite contamination. Another contributing factor was that, participants did not use feed supplements to feed their cows with cereal feeds.

Several thousand people were affected by gastrointestinal distress in an incident in the Kashmir Valley of India in 1987 (Bhat *et al.*, 1989). The toxic effects induced by DON are well characterized in all animal species, with pigs being the most susceptible (Pestka, 2010). DON can affect the immune system, animals' food consumption, growth, reproduction, neuroendocrine signaling and also intestinal function (Pinton *et al.*, 2008; Pestka, 2010; Pinton *et al.*, 2010). In humans, there is historical evidence suggesting that

DON causes acute illness and is frequently associated with outbreaks of gastroenteritis (Pestka, 2010).

The intestine is the first barrier to food contaminants. Following ingestion of mycotoxin-contaminated food or feed, intestinal epithelial cells can be exposed to high concentrations of toxins (Maresca *et al.*, 2008). Highly dividing cells, such as intestinal epithelial cells, are especially sensitive to trichothecenes and the exposure of intestinal epithelial cells to these toxins may alter their capacity to proliferate and to insure a proper barrier function. Exposure to DON can lead to impaired absorption of nutrients and decrease of cell proliferation or differentiation (Awad *et al.*, 2007; Kasuga *et al.*, 1998; Bensassi *et al.*, 2009). The entry of luminal antigens and bacteria, normally restricted to the gut lumen, was demonstrated to be a consequence of the disruption of the barrier function in cell culture models (Maresca *et al.*, 2008) or in mice species (Li *et al.*, 2005).

5.2 Conclusions

The yeast isolates from the study were *G. candidum* being the major contaminant, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. albicans*, *C. kefyr*, *C. krusei*, *Rhodotorula spp.* and *Sacharomyces cerevisiae* are *mursik* contaminants and all these organisms have been known to be among the major cause of various opportunistic mycoses in immune suppressed patients.

Detection of Internal transcribed spacer region (ITS) and Beta tubulin (Bt) genes indicated the presence of fungi of the genus, *Aspergillus*, *Penicillium*, and *Fusarium* which are known spore forming fungi. This therefore means that they are among the contaminants of raw milk used to produce *mursik* and *mursik* leading to mycotoxin contamination causing mycotoxicoses in consumers.

Aflatoxin is the major contaminant of *mursik* and the level of contamination (mean 4.6 ppb) surpassed the recommended limits of 0.05 ppb as per EU and FDA regulations.

5.3 Recommendations

Microbiological standards on milk and milk products should be put in place for the safety of the consumers and effect of *mursik* on mycotoxigenic moulds should be determined.

Education and training should be given to farmers on milking, storage and maintaining milk containers for proper handling procedures to reduce contamination though there is no documentation showing lack of inadequate education.

Regular studies are required for proper establishment epidemiology of mycotoxin contamination in various foods for the formulation and implementation of country's mycotoxins regulatory limits.

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APPENDICES

APPENDIX I: QUESTIONNAIRE:

REFERENCE _____

SUB-LOCATION _____

Tick (✓) in the box accordingly.

Do you ferment milk? Yes No

Where do you obtain fresh milk? Own cows Buy

Do you add little of fermented milk (mursik) to start fermenting your milk? Yes
No

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

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

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

Do you boil milk before fermenting? Yes No

What do you use to ferment milk? Calabash Plastic container

Do you add the charcoal like material to the milk (wo'sek)? Yes No

For how long do you leave the milk to ferment? 2 days 3 days - 4 days

Give us the interval of adding milk to your continuous milk fermentation. 1 day 2 days

Do you wash containers before the next fermentation? No Yes

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

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

If yes, where do you obtain them and how do you store them.....

Do you have a sample of the feed Yes No?

Can I buy ¼ kilo of the feed Yes No? This is to determine whether the feeds are contaminated with mycotoxins which will be transferred to milk.

Translated version to the local language (Kipsigis).

TEBUTIK:

KOITET _____

KOKWET _____

Irechche chego? woi acha

Inyorchini ano chego? Tukyuk oale

Itesyini mursik tuten keiwet asiireche? Woi achicha

Igochinen moita ano? Munandet ne shebat lon puch ketit kel

Iundai ano tukuk che irechen chego? Beek che lale beek che koitit

Igonoren ano tukuk ab chego? Got ne gimenye choge

Ignore ano chego? Gotab chego legikwany'en choge ple kirwe

Igere chego che ga kigee? woi acha

Iyoi chego kotomo iriech? woi acha

Iboishen ne irech chego? sotet nbiret

Iteshini wo'sek cheko? Woi achicha

Irieche chego betusiek ata? Ae'ng somok ang'wan

Ibe kasar netyan asiteshi cheko sotet nemi chego cherechokse? Betut egenge betusiek
aeng'

Iuni tukuk chebo chego kotomo inam iriech alak? woi acha

Ibaen ne tuka che gikee? marurek suswek en tiriita

Tos ibaen amitwagik che giale? Woi acha

Goti tinye, inyorchini ano? Ago igonortoi ano?

Itinye tuten? woi acha

Amuche ayal tuten? Woi acha

APPENDIX II: INFORMED CONSENT DOCUMENT

PROJECT TITLE: Mycotoxin contamination and molecular characterization of mycotoxigenic fungi in traditionally fermented (mursik) milk in Soliat Location which constitute Kongeren, Soliat, Kamasega and Motero Sub locations, Kericho County.

INVESTIGATORS:

1. Mr. Talaam Kiplangat Keith from Institute of Tropical Medicine and Infectious Diseases/Jomo Kenyatta University Agriculture and Technology.
2. Dr. Bii C. Christine from Kenya Medical Research Institute (KEMRI).
3. Prof. Ng'ang'a Zipporah from Jomo Kenyatta University of Agriculture and Technology.

INTRODUCTION:

My team and I (Talaam Kiplangat Keith, the lead investigator and a student) are researchers from Institute of Tropical Medicine and Infectious Diseases, Kenya Medical Research Institute and Jomo Kenyatta University of Agriculture and Technology involved in research studies aimed at improving the health and wellbeing of Kenyans. We are conducting a research on **'Mycotoxin contamination and molecular characterization of mycotoxigenic fungi in traditionally fermented (mursik) milk in Soliat Location which constitute Kongeren, Soliat, Kamasega and Motero Sub locations, Kericho County'**. This is a study or research whereby your participation is based on your willingness and no one is going to force you to be part of the study. You are free to be part or refuse to be part of the study.

PURPOSE OF THE RESEARCH

We intend to determine the fungi and their toxins in *mursik*. We will collect samples of complete fermented milk (mursik) to check for fungi and mycotoxin contamination. This will make it possible for us to determine the fate of mycotoxins during fermentation. We will do it At Kenya Medical Research Institute. Anyone who is going to participate in the study will be part of it for a period of three months.

PROCEDURES:

We request you to allow us to buy a cup ('namba nane') of your *mursik*. We will test your samples for the presence of mycotoxins and fungi. For fungi tests, we will make them grow by placing a small amount of mursik (100µl) on a medium where the fungi will grow. If the fungi will grow, we will isolate and characterize them to know the types and if they are capable of producing mycotoxins. We will determine the presence and levels mycotoxins using test kits called Envirologix kits. Some other information will be asked through the questionnaire whereby you will fill it accordingly. The test will run for three months.

BENEFITS

The study will tell us if the milk is contaminated with mycotoxins and if milk is actually contaminated, we will get to advise you not to use or sell to other people and teaching sessions will be done milk on how to handle milk and avoid the contamination.

RISKS:

There will be no risks in allowing us to test your *mursik*.

CONFIDENTIALITY:

In this study, all samples will be given code numbers and will not bear the names of the fermenters. The findings of this study will be kept confidential on coded records that will not be traced to individuals. In case a participant wants to have feedback his/her phone

numbers will be taken and the records containing the participant's information will be only accessed by the principal investigator.

COMPENSATION:

There will be no direct compensation for participation in the study. We will only buy a cup of your fermented milk.

STORAGE, EXPORTATION OF SAMPLES AND FURTHER STUDIES:

Milk samples collected will be transported to Kenya Medical Research Institute and kept refrigerators to wait for tests where fungal and mycotoxin contamination will be determined. Small amounts of 100ml (aliquot) of the samples will be stored in case further studies will be required and the rest will be autoclaved and discarded.

CONTACT OF THE PRINCIPAL INVESTIGATOR

For any questions you will like to ask, you can do so through the following contacts of the Principal investigator: Talaam Kiplangat Keith. In Nairobi, P.O Box 19464-00202, Nairobi, Kenya or at my home near cattle dip in Soliat location, Kongeren Sublocation, Chelogong village P.O Box 11- 20208, Kiptugumo. Phone number: 0726 462 022 on days and night or E-mail address: keithtalamk@gmail.com

CONTACT OF KEMRI/ERC

For any questions concerning your right of participation, you are free to call the Kenya Medical Research Institute's Ethics review committee through; The Secretary, KEMRI Ethics Review Committee, P.O Box 54840-00200, Nairobi. Telephone numbers: 020-2722541, 0722205901, 0733400003; Email address: erc@kemri.org

CONSENT AND SIGNATURE OPTIONS

I confirm that I understand the information provided to me for the above study and that I have had the opportunity to ask questions. I understand that my participation is voluntary and that I am free to be or not to be part of the study.

I agree to participate in the study and allow my *mursik* to be sampled.

Yes, I would like to be contacted with the results of tests done on my *mursik* (tick the check-box if yes)

Participant's Signature/Thumb print Date

Researcher's Signature..... Date

Researcher's Name: TALAAM KIPLANGAT KEITH

**Translated version of the Informed Consent Document to the local language
(Kipsigis).**

KOGUYET AK NAET AB CHIGILISIONI.

METIT AB BOISIET: Chigilet ab ng'wanet ak kabesiaet ab bobek che gonu ng'wanet en mursik en Soliat Location ne'iume kokwotunwek ang'wan choto go: Kongeren, Soliat, Kamasega ak Motero, en jimboit ab Kericho.

CHIGILIK:

1. Mr. Talaam Kiplangat Keith goyab gotab kipsomaninik chebo barak nebo muswagnetet ak kabotisiet, Jomo Kenyatta.
2. Dr. Bii C. Christine goyab gotab chililet ab kerichek nebo Kenya (KEMRI).
3. Prof. Ng'ang'a Zipporah goyab gotab kipsomaninik chebo barak nebo muswagnetet ak kabotisiet, Jomo Kenyatta.

MWAET AB'GE EN NW'OGINDO:

Gibagenge nenyu ak ane (Talaam Kiplangat Keith, chigilindet ne-o ago kipsomaniat) go chigilik goyab gotab kipsomaninik chebo barak nebo muswagnetet ak kabotisiet, Jomo Kenyatta ak gotab chililet ab kerichek nebo Kenya (KEMRI) goyae chigilet chetogyi'nge gotoror sabet ab kipkosabei en Kenya. Kiyae Chigilet ab ng'wanet ak kabesiaet ab bobek che gonu ng'wanet en mursik en Soliat Location ne'iume kokwotunwek ang'wan choto go: Kongeren, Soliat, Kamasega ak Motero, en jimboit ab Kericho. Niton ko chigilet ne mache biik ko'gonge en chameny'wan ako iyanat koesio chi agetukul komat'kobota boisioni.

AMUNE NEBO CHIKILISIONI:

Gitakyinige gecheng' bobek ak ngw' Janet chegonu bobek en mursik. Kiyumi mursik tuten asi kechigilik meng'isiet ab bobek ak ngw' Janet en chego. Boisioni tugul keyae en gotab chilisiet nebo kerichek nebo Kenya. Chi agetugul negonu'ge, koboto boisioni en kasartab arawek somok.

OLEGIYAITO BOISIONI:

Kisome keyal mursi chebo gikomet 'namba nane'. Ipgechigili gotkomi bobek ak nyw' anet nyw' a. chigilet ab bobek, ko ba keibe mursik tuten ak kerangyi amitwogik che imuch koyai bobek choto korut. Kotkorut, gi'cheru ak keger keboisien muswagnetet neimuche koborwech kotgomuche bobekyuton kogon ngw' anet. En ngw' anet gechikili chang' indo keboisien muswaknotet nebunu got ab Envirologix. Ara ng' olit alak gomi sogkyot age nebendoti ak sokyondeni. Boisioni goibe kasar nebo arawek somok.

BOROTET:

Chigilisioni komawawech kotkomi nyw' anet ab bobek ak bobek che konu nyw' anet. Ko kinetige kele many' olu keboisien anan kigichi chi agetugul anan gelada ak kanetisiet nebo olegiribto chego amatiny bobek.

NG'OYONDIT:

Mami ng' oyondit agetugul en mursik che kichikili.

UNG'OTIET:

En chigilisioni, mujrsik chegiyumi koma kibartai en kaina nebo chi. Mursik kigochin sirutik noton chenoyotin koba koitasiek. Walutik alaktugul kegonori en ung' ot ak ityitn chigilinet ne-o. Chi agetugul nemache walutik gogonu oret ne kimuchi keityin ana kogon koitosiek kwak cheba 'simoinik'.

WALEIWET:

Mami waleiwet agetugul en chi agatugul nemi chigilishoni. Kiale mursik kityo chebo kigombet 'namba nane'.

KONORET, IBET AB MURSIK AK CHIKILISIET NEBO BETUSIEK CHEBWONE:

Mursik kiyumi ak ke-ib koba gotab chigilisiet nebo kerichok (KEMRI) NEBO KEGONORI EN MUSWAGNOTET NEIGOTITI KOGONYE CHIGILISIET. Kigonorri tuten kogany chigilet nebo betusiek chebwone ak alak kebele.

ITY'INET AB CHIGILINDET NE-O:

En tebutik alaktugul che imuche iteb chi agetugul iteben chigilindet ne-o, imuche iyai kobun posta: Talaam Kiplangat Keith. P.O Box 19464-00202, Nairobi, Kenya anan go en gaa yenegityin ak dip nebo tuga en kokwet ab chelogong sublocation nebo Kongeren en Soliat location P.O Box 11-20208, Kiptugumo. simoit: 0726 462 022 anan ko oret nebo E-mail: keithtalamk@gmail.com

ITY'INET AB BIIK CHE TONONCHIN IMANDAB KIPKOSABEI NEBO KEMRI:

En tebutik alaktugul che imuche iteb chi agetugul agobo imanda'ng'ung iteben che tononchin imandab kipkosabei nebo kemri kobun sirindet ibune posta; The Secretary, KEMRI Ethics Review Committee, P.O Box 54840-00200, Nairobi. simoit: 020-2722541, 0722205901, 0733400003; Email: erc@kemri.org

KAYANET AK SI'YET

Ane ayani ale ng'alek che kagigonon en chigilisioni ko akuitosi ako kagekonon kasarian ateb tebutik. Akuitosi ale ami en chigilisioni ko makisigyin chi ako atinye kasar koboton chigilishoni.

Ane ayani goboton chigilisioni ak akonu mursik.

Woi, amache keguron kemwoiwon walutik chebo mursik (inde si'yet)

Si'yet..... Betut

Si'yet ab chigilindet..... Betut

Chigilindet: TALAAM KIPLANGAT KEITH

APPENDIX III: ANALYTICAL PROFILE INDEX (API); YEAST FERMENTATION OF SUGARS.

Analytical Profile Index (API) TESTS					
Fungi	Fermentation of sugars				
Yeast spp.	Glucose	Maltose	Galactose	Sucrose	Lactose
<i>Geotricum candidum</i>	-	-	-	-	-
<i>Sacharomyces cerevisiae</i>	+	+	+	+	-
<i>Candida albicans</i>	+	+	+	-	-
<i>Candida parapsilosis</i>	+	-	-	-	-
<i>Candida kefyr</i>	+	-	+	+	+
<i>Candida glabrata</i>	+	-	-	-	-
<i>Candida tropicalis</i>	+	+	+/_	+	-
<i>Candida krusei</i>	+	-	-	-	-

Key; + showed that there was fermentation, +/_ no clear fermentation and - shows no fermentation.

APPENDIX IV: AFLATOXIN QUANTIFIED LEVELS.

Aflatoxin Quantification		
Toxin level (ppb)	Frequency	% of total
0	2	1.0
2.5	1	0.5
2.6	1	0.5
2.8	1	0.5
2.9	2	1.0
3	3	1.5
3.1	4	2.0
3.2	5	2.5
3.3	1	0.5
3.4	5	2.5
3.5	3	1.5
3.6	4	2.0
3.8	8	4.0
3.9	5	2.5
4	5	2.5
4.1	5	2.5
4.2	5	2.5
4.3	12	6.1
4.4	3	1.5
4.5	8	4.0
4.6	5	2.5
4.7	6	3.0
4.8	13	6.6
4.9	9	4.6
5	12	6.1
5.1	8	4.0

5.2	10	5.1
5.3	5	2.5
5.4	12	6.1
5.5	7	3.5
5.6	6	3.0
5.7	3	1.5
5.8	4	2.0
5.9	5	2.5
6	4	2.0
6.1	1	0.5
6.3	2	1.0
6.9	1	0.5
11	1	0.5
12	1	0.5
Total	198	100

APPENDIX V: INVESTIGATIVE VARIABLE RESULTS.

Investigation variables of milk fermentation			
Variable		Frequency	% of total
Obtain milk from own cows		194	100
Milk fermenters		194	100
Use of calabash		194	100
Use of starter cultures	yes	0	0
	no	194	100
Milking cows in the open	Yes	194	100
	No	0	
Washing calabashes using	Hot water	155	80
	Cold water	39	20
Storing calabash and other milk containers	In maize store	144	74
	In living rooms	50	26
Milk storage	In maize store	144	74
	In living rooms	50	26
Covering freshly milked milk.	Yes	19	10
	No	175	90
Use of wo'sek	Yes	194	100
	No	0	
Fermenting time	3-4 days	194	100
	>4days	0	
Time interval for milk addition on continuous cultures	1 day	194	100
	2days	0	
Cleaning calabashes	Yes	194	100

before the next batch	No		
Use of commercial feeds	Yes	0	
	No	194	100
Boiling of milk before fermentation	Yes	23	12
	No	171	88
Feeding cows by use of nappier grass and grazing in the open fields	Yes	194	100

APPENDIX VI: ETHICAL CLEARANCE