ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCES

SCHOOL OF ALLIED HEALTH SCIENCES

DEPARTMENT OF MEDICAL LABORATORY SCIENCE

Status of Aflatoxin M1 and bacteriological quality of pasteurized milk commercialized in Addis Ababa city, Ethiopia

By: Million Sleshi Weldemedhin (BSC)

Advisors: Mr. Kassu Desta (MSc,PhD Fellow, Assistant Professor)

Mr. Samuel Kindie (MSc,PhD Fellow, Assistant Professor)

A Research Thesis Submitted to the Addis Ababa University College of Health Sciences, School of Allied Health Sciences, Department of Medical Laboratory Sciences, in Partial Fulfillment of the Requirements for the Degree of Master of Science in Clinical Laboratory Science (Diagnostic and Public Health Microbiology)

Jan, 2018

Addis Ababa, Ethiopia

Addis Ababa University
College of Health Sciences
School of Allied Health Sciences
Department of Medical Laboratory Science

This is to certify that the thesis prepared by Million Sleshi Weldemedhin, entitled: **Status of Aflatoxin M1 and bacteriological quality of pasteurized milk commercialized in Addis Ababa city, Ethiopia** and submitted in partial fulfillment of the requirements for Master of Science degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology Specialty) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the Examining Committee:

Internal Examiner _____ Signature _____ Date_____

External Examiner _____Signature _____Date_____

Advisor _____Signature _____ Date_____

Advisor _____Signature _____Date_____

Chairman of the Department or Graduate program coordinator

_____ Signature _____ Date_____

Acknowledgment

First of all, I would especially like to thank GOD who always led me in triumph through Christ Jesus.

Next, I would like to forward my profound gratitude and deep regards to my advisors Mr. Kassu Desta and Mr. Samuel Kindie for their exemplary guidance, monitoring and welcoming approach and material support throughout the course of this thesis.

I would like to give my long list of thanks to BLESS Agri food Laboratory Services PLC for providing laboratory facilities for this project. I would also like in particular to thank Mr. Belete Beyene, Managing Director of the company, for his in-kind allowing me to conduct this study on the company's property and inspiring me in all activities.

I would like to extend my appreciation to those who helped with various aspects of my project: Mr. Birhanu Legesse, my immediate manager, his kindness and positive thoughts will never be forgotten in my life. Habitamu Gashaw, Henok Ashagrie, Kalelias Agumas, Mulugeta Legesse, Asrat Mihiretie, Yordanos Tesfaye, Bethelhem Mengistu, Geresu Bechere, Dejene Shiferaw Tesfaye Legesse and Adinew Zewdu who all played magnificent roles to this thesis to have its current form.

And I cannot forget to thank my brother Lolassa Hailemichael specifically for lowering my burdens heading here and there.

And of course, I need to thank my life partner, Rediet Tsegaye. Thank you for encouraging me to pursue my master's. No thanks will ever be enough, but I would like to say thank you most of all for your loving support and always having confidence in me. I love you.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AFB1: Aflatoxin B1

AFM1: Aflatoxin M1

AOAC: Association of Analytical Communities

ATCC: American Type Culture Collection

CFU- Colony Forming Unit

CPC: Coliform Plate Count

EAS: East African Standard

ESA: Ethiopian Standards Agency

FAO: Food and Agriculture Organization

FDA: Food and drug Administration of United states

FMHACA: Food, Medicine and Health Care Administration and Control Authority of Ethiopia

HBV: hepatitis B virus

HCC: Hepatocellular Carcinoma

HPLC: High-Performance Liquid Chromatography

IARC: International Agency for Research on Cancer

JECFA: Joint FAO/WHO Expert Committee on Food Additives

ND: Not Detected

PCA: Plate Count Agar

TVBC: Total Viable Bacterial Count

TCC: Total Coliform Count

UHT: Ultrahigh-temperature

UNICEF: United Nations Children's Fund

UNRRA: United Nations Relief and Rehabilitation Administration

USPHS: United States Public Health Services

WHO: World Health Organization

Abstract

Background: Aflatoxin M1 (AFM1) in milk and milk products is considered to pose certain hygienic risks for human health especially infants and children.

Objective: The aim of this study was to assess Aflatoxin M1 (AFM1) and bacterial level in 500 ml pre-packed pasteurized milk samples commercialized in Addis Ababa city, Ethiopia.

Methods: A cross sectional prospective study was conducted during February to December 2017. A total of 65 samples of pasteurized milks of different brands from a variety of supermarkets across Addis Ababa city were analyzed for the presence of AFM1 by immunoaffinity column clean-up coupled to HPLC with fluorescence detection based on the method of AOAC Official Method 2000.08 and bacteriological quality was assessed for TVBC and TCC based on the methods of US FDA and ISO 4832:2006 respectively.

Result: AFM1 was found in 60/65 (92.3%) of the examined pasteurized milk samples by average concentration of 0.382µg/l and most of AFM1 concentration levels range with 95% confidence interval (CI) lays between 0.299µg/l – 0.465µg/l. The concentration of AFM1 in 37/60 (61.7%) of the samples was lower than US FDA limit (<0.5µg/l) and in 56/60 (93.3%) of the samples AFM1 concentration was greater than the maximum tolerance limit (0.05µg/l) accepted by European Union. The bacteriological analysis result revealed that the pasteurized milk samples were substandard in terms of TVBC and TCC. The ranges of TVBC and TCC with 95% CI in pasteurized milk samples were $2.1x10^5$cfu/ml to $5.4x10^5$cfu/ml and $1.6x10^2$cfu/ml to $3.8x10^2$cfu/ml respectively, and E. coli was detected in 4 out of 65 (6.2%) samples.

Conclusion: The present study concluded that serious risks for public health exist from pasteurized milk consumption and the pasteurized milk commercialized in Addis Ababa city is of low quality. Hence we recommend that pasteurized milk products have to be controlled for AFM1 contamination and hygienic quality monitoring programs are urgently needed to ensure that quality pasteurized milk is produced and consumed in the city.

Keywords: Aflatoxin M1, Coliform, E. coli, Pasteurized milk, HPLC

1. Introduction

1.1 Background

Milk is a good source of many nutrients, and it is used extensively even as a main food in many countries. However milk could also be a source of toxic substances such as Aflatoxin M1 (AFM1) and microorganisms. Aflatoxins are a group of structurally related mycotoxins produced by certain species of the genus *Aspergillus*, particularly *A. flavus*, *A. parasiticus* and *A. nomius*, which can grow on a variety of food and feed commodities [1]. Aflatoxin production is influenced by several factors: for example, temperature and humidity [2]. It has been shown that Aflatoxin B_1 (AFB_1) is the most potent hepatocarcinogen of this group of mycotoxins.

Aflatoxin M_1 (AFM_1) is a hydroxylated metabolite of AFB_1 produced by the hepatic microsomal cytochrome P450, and is secreted in the milk of mammals that have consumed AFB_1-contaminated foods [3]. The amount of AFM_1 excreted is directly related to the level of AFB_1 consumed in the feed. The AFB1, once ingested by the animal, is rapidly absorbed by the gastrointestinal tract and is transformed into the metabolite AFM1, which appears in the blood after 15 minutes and is then secreted in the milk by the mammary gland [4].The amount of AFM1 which is found in milk depends on several factors, such as animal breed, lactation period, mammary infections etc. It has, anyway, been demonstrated that up to 6% of the ingested AFB1 is secreted into the milk as Aflatoxin M1 and, because AFM1 is relatively resistant to heat treatments, it is almost entirely retained in pasteurized milk, powdered milk, and infant formula. Moreover, only a limited decrease of AFM1 content has been verified in Ultrahigh- Treatment milk after long storage [5].

The hepatotoxicity and carcinogenic effects of AFB1 have been clearly demonstrated, thus it has long been classified as a group 1 human carcinogen by the International Agency for Research on Cancer (IARC). Initially, the IARC classified AFM1 as a possible carcinogen for humans (group 2b) since toxicological data was limited. However, genotoxicity and cancerogenity of AFM1 have been observed *in vivo*, although lower than those of AFB1, and its cytotoxicity has been definitively demonstrated. As a result of these and other further investigations, the IARC moved Aflatoxin M1 from group 2B to group 1 human carcinogen [6].

This is supported by a number of epidemiological studies done in Asia and Africa that have demonstrated a positive association between dietary aflatoxins and Liver Cell Cancer. In terms of food safety and public health concerns, exposure to AFM_1 through milk products is considered to be a serious problem. It has been experimentally shown to confer high hepatotoxic and mutagenic risk [7].

In order to decrease Aflatoxin risk nearly all developed countries have set the maximum permissible levels of AFM_1 in milk and milk products. Currently the limits of AFM_1 are highly variable, depending upon the degree of development and economic standing of the countries. Some European Community and Codex Alimentarius prescribe that the maximum level of AFM_1 in liquid milk and dried or processed milk products should not exceed 50ng/kg. However, according to US regulations the level of AFM_1 in milk should not be higher than 500ng/kg [3].

There are still several countries, including Ethiopia, that have not yet established regulatory limits for AFM_1 in dairy products. There is very little data in the literature on AFM_1 levels in the milk produced in Addis Ababa, the capital city of Ethiopia. Therefore, it is difficult to estimate the daily intake of $AFM_1$from milk or other dietary sources, thus there is a need to detect and quantify AFM_1 in milk and milk products. Various methods to determine AFM_1 have been developed, including Radioimmunoassay, enzyme-linked immunoassay, and high-performance liquid chromatography (HPLC) [8].

On the other hand, since cow's milk consists of a variety of nutrients such as fats, proteins, minerals, vitamins, carbohydrates and water, it serves as an excellent medium for bacterial growth. Given the appropriate conditions milk can act as a carrier of disease causing microorganisms' transformation from cows to humans [9].

Microorganisms may contaminate milk at various stages of milk procurement, processing and distribution. The health of the cow and its environment, improperly cleaned and sanitized milk handling equipment, and workers who milk cows come in contact with milk due to a number of reasons could serve as sources of microbial contamination of milk .Use of non potable water may also cause entry of pathogens in to milk. It is known that tropical conditions which have a hot, humid climate for much of the year are ideal for quick milk deterioration so pose particular problems because the temperature is ideal for growth and multiplication of many bacteria [10].

Now a day, 500ml pre-packed pasteurized milk in most places in Ethiopia is consumed. Hence, there exists the possibility of consuming pasteurized milk, which has been contaminated with disease causing organisms.

Hygienic quality control of milk and milk products in Ethiopia is not usually conducted on routine basis. Apart from this, door to door raw milk delivery in the urban and peri-urban areas is commonly practiced with virtually no quality control at all levels [10].

Therefore in order to protect the public health, microbiological assessments have an important role to play in the dairy industry. This will also reduce economic losses by the early detection of insufficient processing, packaging or refrigeration.

This study is, thus, planned to assess the status of AFM1 using High Performance Liquid Chromatography (HPLC) method and bacteriological quality of 500 ml pre-packed pasteurized milk commercialized in Addis Ababa-Ethiopia during February 1 to December 15th 2017.

1.2. Statement of the Problem

Currently, pasteurized milk is widely consumed by most people (wide age group) even as meal. Milk has the greatest demonstrated potential for introducing AFM1 into the human diet. Evidence of potential hazardous human exposure to AFM1 through milk and milk products has been shown in several studies. According to Karimi G., epidemiological studies carried out in several parts of Africa and Asia indicates a correlation between exposure to aflatoxins and primary liver cancer [11]. The risks associated with exposure to aflatoxins are enhanced by simultaneous exposure to hepatitis B virus and possibly hepatitis C viruses. Recent studies carried out in West African countries including; Benin, Gambia, and Togo indicate chronic exposure of population groups and fetuses to dietary aflatoxins. More recently, Aflatoxin exposure early in life has been associated with impaired growth, particularly stunting [4]. Therefore, the presence of AFM1 in milk and dairy products may pose a threat, mainly towards children who are considered to be the major consumer of milk and dairy products in many countries [5].

The consumption of milk and milk products by human is quite high, particularly among infants and young children, thereby increasing the risk of exposure to AFM1 [12, 13]. AFM1 is relatively stable during pasteurization, sterilization, preparation and storage of various dairy products [14, 15].

In Ethiopia, Even though the study conducted by ILRI reported that the AFM1 contamination of the Great Addis Ababa's Milk shed is highly contaminated [16]; there is still a large paucity of research based data available on occurrence of AFM1 in pasteurized milk and other milk derivatives.

Internationally, consumption of milk has commonly been associated with food borne illness. Between 1992-2000, 2% of all food borne outbreaks in England and Wales were milk borne, with the majority (52%) attributed to the consumption of milk .In USA where permissions for the sale of milk currently exist in 22 states, 58 outbreaks were attributed to milk between 1978-2000 [17].

The world still faces great problems of food borne diseases associated with contamination of the food supply. World Health Organization estimates that each year there are 1.3 million cases of

active diarrhea in children less than five years in the developing world. A substantial number of these cases are due to microbial contaminated foods. It is also clear that much of foods are lost as a result of spoilage [18].

Milk is one of the food products which are used as a vehicle of disease transmission. Tuberculosis, typhoid fever, dysentery, diphtheria, septic sore throat and other streptococcal infections, staphylococcal toxins, salmonella gastroenteritis, and brucellosis are some of the diseases transmitted by milk, while reports on the microbiological quality and safety of milk and milk products from Ethiopia are scanty [19].

Thus, in this study, status of AFM1 and bacteriological quality of 500 ml pre-packed pasteurized milk commercialized in Addis Ababa city, as one of the majors pasteurized milk producing and commercializing city in Ethiopia is investigated to fill the existing research gap.

1.3 Significance of the study

This study will help producers to have an idea about the quality issue of pasteurized milk related to Aflatoxin M1 and bacterial safety so as to stay competent in the market. It also benefits the consumer to prevent disease by assuring the quality of pasteurized milk they consume. Vendors will develop knowledge about AFM1 and hence this study will guide them to develop good competition. Government regulatory bodies like Ethiopian Standard Agency, Ethiopian Food, Medicine and Health Care Administration and Control Authority and Addis Ababa Health Bureau can use the study result to apply appropriate disease prevention strategies. Moreover, the study will be used as an initial data for future researches on AFM1 and bacterial contamination of pasteurized milk. It helps to alert people consuming pasteurized milk in such a way that consumers should know which to consume.

2. Literature review

2.1. Overview of AFM1 in Milk

Milk is the most important source of calcium and phosphorus of human body and due to having essential amino acids, has an important status in supplying the body's protein needs. Studies have shown that there is a close relationship between consumption of milk and health status of people in terms of efficiency, Intelligence quotient (IQ), reducing the risk of infectious diseases, regulation of metabolic activities, decreasing blood pressure, increasing beneficial blood lipids (High-density lipoprotein), preventing from colon cancer and osteoporosis [20].

Milk is a key contributor to improve nutrition and food security particularly in developing countries and play a significant role in reducing poverty and malnutrition [20]. However milk, as a liquid, is a highly variable product that rapidly loses its quality and spoils if not to be treated. Since milk may be processed in numerous ways, the effects of storage and processing on stability and distribution of AFM1 are of great concern. As to the effect of cold treatment detectable AFM1 decreased by 11 to 25% after 3 days at 5ºC, 40% after 4 days at 0ºC, and 80% after 6 days at 0ºC [21]. Whereas, freezing at -18ºC for 30 days resulted in an apparent loss of 14%, with 85% lost after 53 days [22], and less degradation of AFM1 at -18ºC with insignificant loss after 53 days [23]. As to the effect of heating contradictory data have been reported, some reported that various heat–time treatments caused reductions in the AFM1 concentrations of milks between 12% and 40% [21]. While others studied the effect of various heat treatments on AFM1 content of cow's milk and reported that sterilization of milk at 121 °C for 15 min caused 12.21% degradation of AFM1 [24], whereas boiling decreased AFM1 by 14.50%. They concluded that destruction of AFM1 depends on time and temperature combination of the heat treatment applied. In an investigation conducted it was observed that pasteurization caused a decrease in the level of AFM1 at the rate of 7.62% [25]. While other showed that pasteurization can partially reduce the amount of AFM1 in milk [26]. However, some reports showing that aflatoxins are stable during heat-treatments such as pasteurization and sterilization [27]. AFM1 distribution in milk is not homogeneous. Cream separation can affect AFM1 distribution, since 80% is partitioned in the skim milk portion [28] because of AFM1 binding to casein [29]. An amount of 30% of AFM1 is indeed estimated to be associated with the nonfat milk solids and in particular

with casein. Contradictory data have been reported on the influence of milk concentration on AFM1. Some reported no losses of AFM1 [30], whereas some authors observed losses ranging from 60 to 75% following milk concentration [31].

Aflatoxins are one of the major etiological factors in the development of hepatocellular carcinoma, and more recently associations between childhood Aflatoxin exposure and both growth faltering [4] have been reported. According to worldwide regulations for mycotoxins in food and feed compiled by the Food and Agriculture Organization of the United Nations, 60 countries have already established regulatory limits for AFM_1 in raw milk and milk products. The report also indicates that the limits vary from ND (not detectable) to 15µg/l [3]. Numerous studies have been conducted on the effects of processing on the concentration of Aflatoxin M1 in milk, the results of which were variable. Most studies show that the concentration is not appreciably reduced by heat treatment or by processing yogurt, cheese, cream, milk powder and butter. Due to the high toxicity of Aflatoxin M1, the European Community (EC) established the maximum level of 0.05 µg /l for Aflatoxin M1 in liquid milk. This limit is also applicable to milk products, which are dried or processed, taking into account the concentration caused by the drying process or by other form of processing. However, the Codex Alimentarius, Joint Expert Committee on Food Additives (JECFA) has established an acceptable level of risk at 0.05 µg kg^{-1} for Aflatoxin M1 in fluid milk. The level for AFM1 in milk and dairy products in the United States is tenfold higher (0.50 µg /kg) than the current level in the EC [3].

Several studies conducted in Iran have shown the presence of AFM1 in milk and dairy products. A study carried out by Karimi G, showed that 82% of UHT milk samples were contaminated with AFM1 in Tehran, while 100% of contaminated samples had levels exceeding 50ng/l (EC) [11]. In the city of Shiraz, Alborzi A and his colleagues examined over six hundred pasteurized milk samples, in which AFM1 was found in 100% of the examined samples and 17.8% had an AFM1 level greater than the maximum tolerance limit accepted by the EC [32].

In further studies carried out by Oveisi MR and his colleagues, in Babol and Oveisi in Tehran, 100% of milk samples were contaminated and the level of toxins in 78% of the examined samples was higher than the EC accepted limit [33]. On the other hand, in Uremia according to [34] all pasteurized milk samples were lower than the EC standard.

Table 2.1: Maximum limits for Aflatoxin M1 in milks in various countries

Mycotoxin	Country	Maximum limit (µg/kg or µg/l)	Food
Aflatoxin M1	Sweden	0.050	Liquid milk products
	Austria	0.050	Milk
	German	0.050	Milk
	Netherlands	0.020	Butter
		0.200	Cheese
	Switzerland	0.050	Milk and Milk products
		0.250	Cheese
	Belgium	0.050	Milk
	USA	0.50	Milk
	Czech Republic	0.1	Children's Milk
		0.5	Adult's Milk
	France	0.03	Children's Milk
		0.05	Adult's Milk
	Turkey	0.05	Milk and Milk products
		0.25	Cheese

Adapted from CREPPY, 2002

In a recent study carried out by Heshmati A. and Milani M., in Tehran, 55.2% of 210 UHT milk samples, AFM1 was detected in concentrations between 8 ng/l and 249 ng/l; and 33.3% of samples were higher than the maximum tolerance limit (0.05 µg/l) accepted by some European countries [35].

According to Amer A. and Ibrahim M. in Egypt, Aflatoxin M1 contamination have been reported as 52.6% of examined raw milk samples were exceeding the European Commission regulation [36].

Report from Kang'ethe EK. and Lang'a showed that in Kenya, Seventy two percent (315/439) of the milk from dairy farmers, 84% (71/85) from large and medium scale farmers and 99% (88/89) of the pasteurized marketed milk were positive for Aflatoxin M1, and 20%, 35% and 31% of

positive milk from dairy farmers, medium and large scale farmers and market outlets respectively, exceeded the WHO/FAO levels of 0.05µg/Kg [37].

In a recent study conducted by Barbara S. and his colleagues, Aflatoxin contamination in the Greater Addis Ababa of Ethiopia milk shed shows that over 90% of the milk samples contained Aflatoxin M1 levels that exceeded the EU limit of 0.05 µg/l and reported the prevalence and concentration of AFM1 in milk in this milk shed is among the highest reported in the world. And also there are very high levels of Aflatoxin contamination in dairy feeds in the greater Addis Ababa milk shed [16].

2.2. Bacteriological Quality of Pasteurized Milk

Milk is synthesized in specialized cells of the mammary gland and is virtually sterile when secreted into the alveoli of the udder [38] and may be contaminated during milking and handling with equipment, personnel and environmental sources and may contain pathogens [39]. Pasteurization is the widely adopted milk process to ensure completely destruction of all pathogenic and spoilage microorganisms, commonly found in milk and inactivation or reduction of other non pathogenic spoilage bacteria and certain undesirable enzymes to safeguard the food value of milk [40].

FAO/WHO defined pasteurization as "A microbiocidal heat treatment aimed at reducing the number of any pathogenic microorganisms in milk and liquid milk products, if present, to a level at which they do not constitute a significant health hazard [41]. Pasteurization conditions are designed to effectively destroy the organisms Mycobacterium tuberculosis and *Coxiella burnetii*. Initially, pasteurization conditions were devised to inactivate *M. tuberculosis* [42]but subsequently, *C. burnetii* appeared as the most heat-resistant organism present in the milk and therefore, pasteurization was redesigned to achieve at least a 5-log reduction of *C. burnetii* in whole milk [43]. The High Temperature Short Time pasteurization kills 99.999% of pathogens [44] and is effective in reducing the viable population of *Mycobacterium avium* subsp. *paratuberculosis* (4-5 log) but its efficacy depends on the total viable concentration [45]. [46] Noted greater thermal destruction (7.7 vs. 5.0 logs) due to pasteurization of Ultrahigh-temperature milk inoculated with higher concentrations (10^8 vs.10^5 CFU mL^{-1}) of *M. paratuberculosis*, regardless of time-temperature combinations adopted. During pasteurization

the initial rapid decline in population of *M. paratuberculosis* in cow's milk is not due to thermal death but attributed to aggregation of cells into clumps [47]. Pasteurization efficiency can be determined by Phosphatase test. Alkaline phosphatase, an enzyme naturally present in milk of all mammals have a thermal resistance greater than that of the most heat resistant non-spore-forming pathogens commonly found in milk [48] and hence, its destruction confirms proper pasteurization [49]. Positive Phosphatase activity is the indicative of inadequate pasteurization or contamination of pasteurized milk with raw milk or post-process bacterial contamination [50].

Bacteriological analysis of pasteurized milk indicated presence of pathogens like *Staphylococcus* sp., *Salmonella* sp. [51], Coliform from India [52], *Salmonella* from Nigeria [53], *Enterobacter* spp., *Escherichia coli* from Jamaica [54], *Staphylococcus aureus* from Brazil [55], coliform and *B. cereus* from Kuwait [56] and *E. coli* and *S. aureus* from Iran [57] noted complete deactivation of phosphatase and *Salmonella* sp. but presence of coliform in 57.5% samples of pasteurized milk from Brazil. Incidence of pathogens in pasteurized milk [58-60] and food-borne outbreaks due to inadequate pasteurization or post-pasteurization contamination [61] have been reported.

Shelf life of pasteurized milk is influenced by the quality of the raw milk [62], duration of storage of raw milk prior to processing, the heat-treatment employed, concentration of heat-resistant microorganisms, extent of post pasteurization contaminants, packaging system adopted, post-pasteurization storage conditions [63] and effect of light [62].

According to Sourav K. and his colleagues, there are several reasons for the occurrence of bacterial contamination in the pasteurized milk samples such as defect in pasteurization machinery, to survive even after pasteurization, and contamination in the post-pasteurized process due to poor processing and handling conditions and/or maintenance of substandard hygienic practices by working personnel [64]. The TVBC (total viable bacterial count) of the pasteurized milk samples from different locations in Bangladesh was ranged from 1.1×10^2 to 1.8×10^3 cfu/ml, slightly lower than that recommended by the Bangladesh Standards and Testing Institution (not exceeding 20,000 cfu/ml) [65,66] while Dey S. and his colleague, found the bacterial count in pasteurized milk samples in between 7.5×10^7 to 1.24×10^8 cfu/ml [67].

Coliform bacteria are supposed to be absent in pasteurized milk as they cannot survive the pasteurization temperature. Because of the defect in pasteurization process or post pasteurization contamination which includes contamination in packaging materials, defects in pipe lines, TCC may be detected in the pasteurized milk samples [68, 69].

According to Ethiopian Standards Agency, pasteurized milk shall not be offered for sale if a total plate count is above 100,000 cfu per ml and if more than 10 per ml for non faecal coliforms and if not nil for faecal coliforms per ml [70].

Table 2.2: Bacteriological grades for pasteurized milk in Ethiopia

Parameters	**Maximum limit/ml**	**Grade**	**Food**
Total Plate Count	< 50,000	Very good quality	Pasteurized Milk
	50,000-100,000	Good quality	Pasteurized Milk
Faecal Coliform	Nil		Pasteurized Milk
Non faecal Coliform	Not more than 10		Pasteurized Milk
Salmonella	Nil		Pasteurized Milk

Adapted from ES 3462-2009

A study conducted by Aberra A. in and around Addis Ababa, showed that Aerobic plate count conducted on pasteurized milk collected from milk processing plants ranged from $1.2x10^3$ to$5.2x10^5$ cfu/ml and 0 cfu/ml to $2.27x10^4$ cfu/ml for *feacal coliforms* and 0 cfu/ml to $2.2x10^4$ cfu/ml for *Escherichia coli* [71].

3. Objectives

3.1. General Objective

- To assess the level of AFM1 and bacterial quality in 500 ml pre-packed pasteurized milk commercialized in Addis Ababa, Ethiopia.

3.2. Specific objectives:

- To assess the Aflatoxin content of 500 ml pre-packed pasteurized milk from different brands found in Addis Ababa market.
- To investigate whether the concentration of AFM1 in 500 ml pre-packed pasteurized milk commercialized in Ethiopia is within the acceptable level for consumption or not.
- To examine the bacterial load in 500 ml pre-packed pasteurized milk from different brands found in Addis Ababa market.

3.3. Hypothesis:

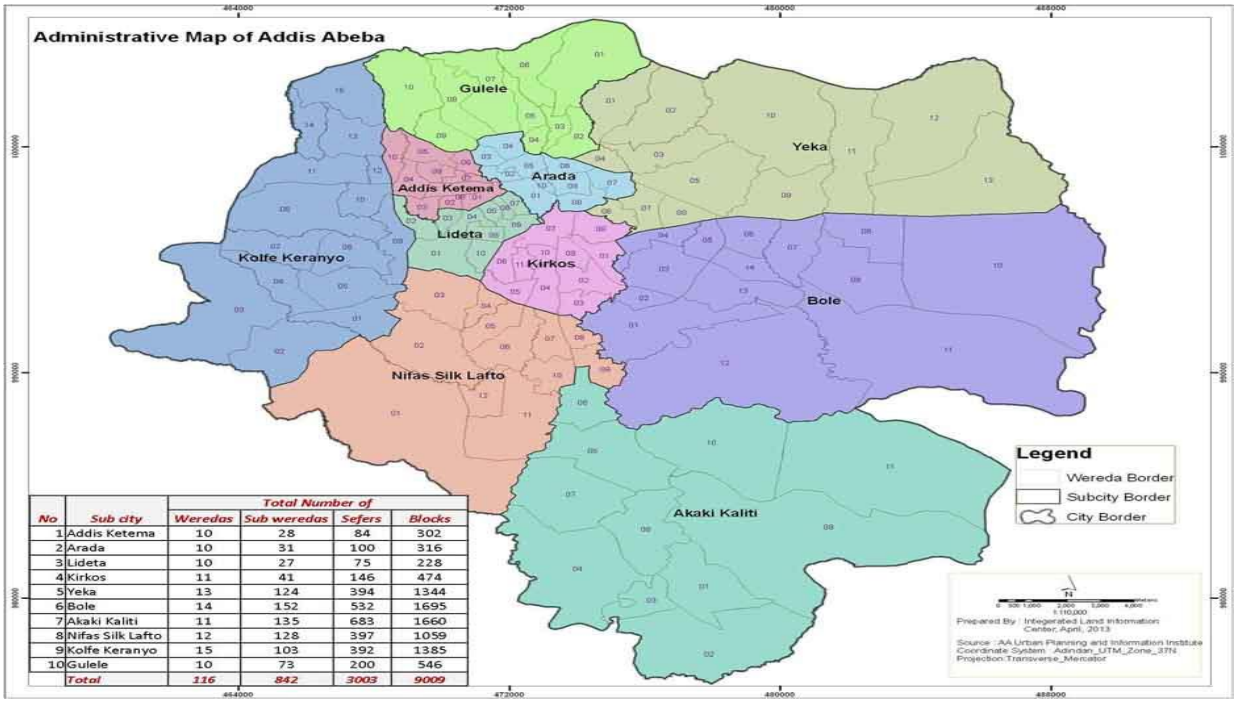
AFM1 concentration and bacteriological quality of 500 ml pre-packed pasteurized milks commercialized in Addis Ababa city is in accordance with international standards.

4. Materials and Methods

4.1. Study site and Period:

The study was conducted in Addis Ababa City, Ethiopia from February 1 to December 15th 2017. Addis Ababa has 10 sub cities having woredas in each of the sub cities (*Figure 4.1*). Addis Ababa is a major trade center for pasteurized milk with many cottage industries that process raw cow milk. In addition, the addresses written on packaging of pasteurized milk products indicate the site of their establishment; which supports the evidence that most processers of pasteurized milk are found in Addis Ababa. This clearly indicates that there is a high demand of pasteurized milk in Addis Ababa, hence, it was chosen as a study site.

Samples were collected from all margins of the city and analyzed at Bless Agri Foods Laboratory (Accredited laboratory in ISO 17025 requirements for nearly 20 chemical and microbial parameters; sister company of Hilina Enriched Foods PLC). The laboratory is established at the heart of Legetafo town, 20km far away from Addis Ababa to the North East.



No	Sub city	Total Number of			
		Weredas	Sub weredas	Sefers	Blocks
1	Addis Ketema	10	28	84	302
2	Arada	10	31	100	316
3	Lideta	10	27	75	228
4	Kirkos	11	41	146	474
5	Yeka	13	124	394	1344
6	Bole	14	152	532	1695
7	Akaki Kaliti	11	135	683	1660
8	Nifas Silk Lafto	12	128	397	1059
9	Kolfe Keranyo	15	103	392	1385
10	Gulele	10	73	200	546
	Total	*116*	*842*	*3003*	*9009*

Figure 4.1: Administrative Map of Addis Ababa City

(Source: - American Academy of Special Education Professionals, 2013)

4.2. Study design

A cross sectional prospective study was conducted at BLESS Agri Food Laboratory Services PLC to assess the concentration of AFM1 and bacterial quality in pasteurized milk commercialized in Addis Ababa, Ethiopia.

4.3. Source population

The source population was all 500 ml pre-packed pasteurized milks of different brands commercialized in different supermarkets in Addis Ababa city, Ethiopia during the study period.

4.4. Study population

The study population was those the 500 ml pre-packed pasteurized milks of different brands which have been registered at FMHACA commercialized at different supermarkets in Addis Ababa city, Ethiopia that fulfill the inclusion criteria during the study period.

4.5. Inclusion criteria

The inclusion criteria was any marketed 500 ml pre-packed pasteurized milk of brand registered at FMHACA from any supermarket in Addis Ababa city, before expiry, shipped in the right temperature and time to BLESS Agri Food Laboratory Services PLC during the study period.

4.6. Study variables

4.6.1. Dependent variables

- Concentration of AFM1
- Level of contamination of TVBC
- Level of contamination of TCC/*E.coli*

4.6.2. Independent variables

- Type of pasteurized milk brands

4.7. Sampling technique

According to a data from FMHACA, currently there are about thirteen (13) registered 500 ml pre-packed pasteurized milk producers in Ethiopia. A total of Sixty five (65) samples of 500 ml pre-packed pasteurized milk of different brands from a variety of supermarkets across Addis Ababa city were randomly collected for the analyses so that each brand was tested five times (i. e five samples from each brands of different batch). All the thirteen brands were included in order to ensure that the survey is representative of the supply of the products to consumers in Addis Ababa. Before sampling was employed, sampling sites were divided in to five directions for ease of sampling; Central, North, South, East, West, so as to minimize missed brands. 500 ml of each type of pre-packed pasteurized milk brands was taken by lottery method from the respective supermarket. Each batch of the pasteurized milk brands were collected in three to four weeks interval.

4.8. Sample Collection and Transportation

Samples were collected within an ice box under aseptic conditions and transported to BLESS Agri food Laboratory Services PLC at 4^{o}C and analyzed within 6 hr of sampling.

4.9. Data Collection procedure

Data was collected using pre-developed template containing all information regarding AFM1 analysis and bacterial quality examinations (Total Bacterial Count, Total Coliform Count and testing for *E. coli*) in pasteurized milk samples. Sample code, time of collection, place of collection, type of laboratory tests and all necessary information were filled.

4.10. Laboratory Methods

4.10.1. Determination of AFM1

Pasteurized milk samples were analyzed for the presence of AFM1 using an immunoaffinity column for clean up and HPLC with fluorescence detection for determination based on the method of *Association of Analytical Communities* (*AOAC) Official Method 2000.08*.

4.10.1.1. Clean-up by Immunoaffinity Column Chromatography

Briefly, 100 ml of pasteurized milk samples warmed at 37^0Ć and centrifuged at 2000 rpm for 15 min. Then the fat layer was removed completely and the skim milk was filtered through Whatman filter paper. The filtrate was gathered in flask and loaded into immunoaffinity chromatography column. Then 50 ml of the filtrate was taken in a syringe barrel which was attached with immunoaffinity columns (IAC). The test portion was passed at the flow rate of 2–3 ml/min. Two times of 10 ml purified water was passed through the column at a rate of 2–3 ml/min. AFM_1 was eluted with 4 ml of HPLC grade acetonitrile and collected in a glass tube. The gentle stream of nitrogen was passed to evaporate the eluate to dryness and the residue was dissolved in 1 ml of mobile phase which is Acetonitrile–water (25:75 v/v).

Figure 4.2: Immune affinity column fitted with syringe barrel (Source: Generated during analysis, 2017)

4.10.1.2. LC Determination with fluorescence detection

The AFM1 content was determined using an Agilent HPLC with 1260 Infinity Automatic Liquid Sampler (G1329B). The flow rate was set at 1 ml/min pumped using the Agilent 1260 Infinity Quaternary Pump (G1311C). The injected sample (50 µl) was passed through the Agilent 1260 Infinity Thermostatic Column Compartment (G1316A) holding an Agilent ZORBAX SB-C8 Reversed Phase Column through to an Agilent 1260 Infinity Florescence Detector (G1321C) set at 365 nm Excitation wavelength and 435 nm Emission wavelength.

The system was interfaced, via network chromatographic software, to a personal computer for instrumentation control, data acquisition and processing. Standard solutions AFM_1 with concentrations of 0.05, 0.1, 0.25 and 0.5 ng/ml in acetonitrile were used to obtain the calibration curve. The retention time for AFM_1 was 10 min.

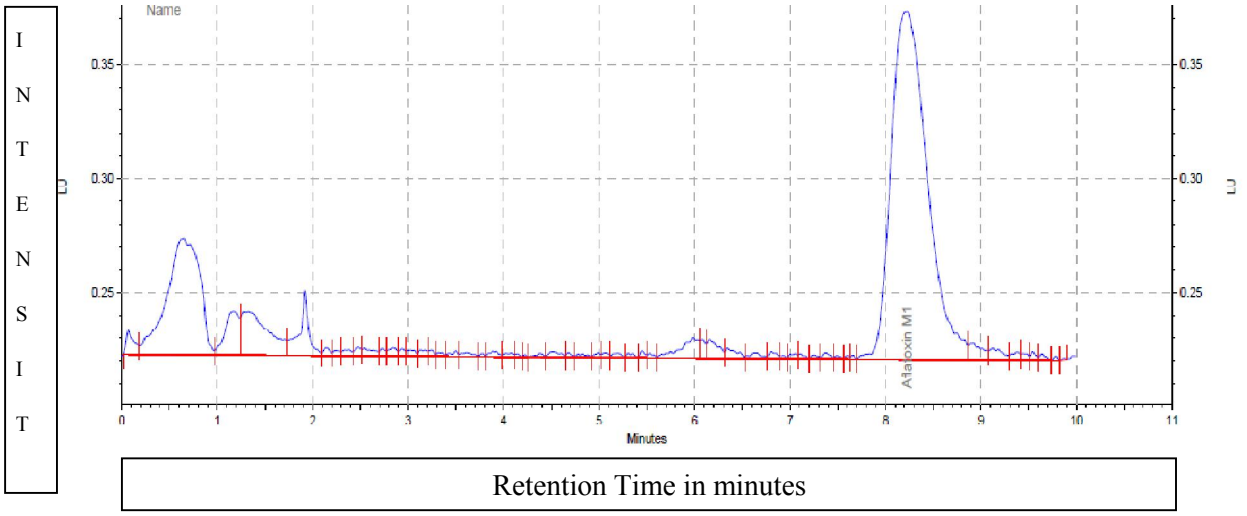


Figure 4.3: HPLC Chromatogram of 0.5ng/ml AFM1 Standard Solution with a retention time of approximately 8.155 minutes (Source: Generated during analysis, 2017)

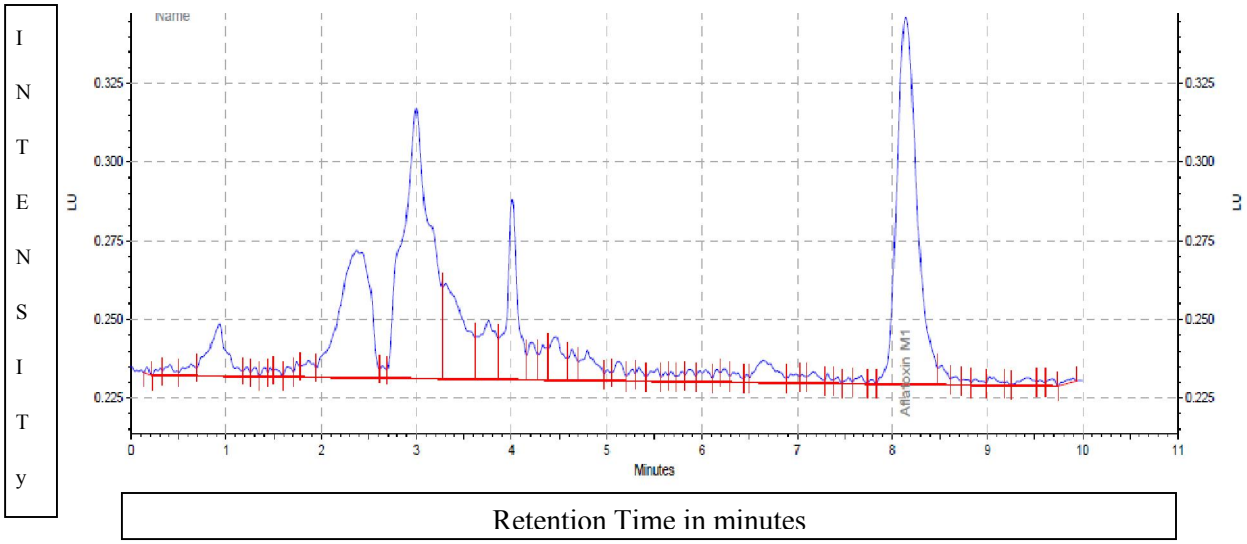


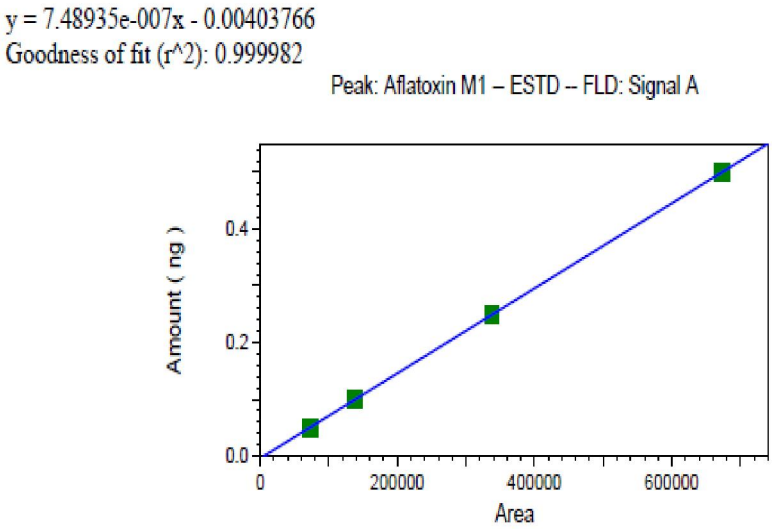
Figure 4.4: HPLC Chromatogram of Pasteurized Milk sample Contaminated with 0.091 µg/l Aflatoxin M1 with a retention time of approximately 8.146 minutes (Source: Generated during analysis, 2017)

4.10.1.3. Method Validation

AFM1 was analyzed with the already validated method by BLESS Agri-Food Laboratory Services PLC, but this had been verified by performing recovery tests.

4.10.1.3.1. Linearity

The standard solutions of concentration from 0.05ng/ml to 0.5ng/ml AFM1 were used to find calibration/standard curve as described by the following regression equation: y= 7.48935e-007x-0.00403766 where y = amount and x= area of AFM1. The results showed the linearity of the standard curve over the range studied. The coefficient of determination (R^2) was 0.999982. *Figure 4.5* gives the calibration curve of standard solutions of AFM1 with concentrations of 0.05, 0.1, 0.25 and 0.5ng/ml by HPLC analysis.



	Level 1	Level 2	Level 3	Level 4
Amount	0.05	0.1	0.25	0.5
Area	73482	138138	338060	673592
RF	6.804387469 04004e-007	7.23913767392 028e-007	7.395136957936 46e-007	7.422891008206 75e-007

Figure 4.5: Calibration curve of standard solutions of AFM1 with concentrations of 0.05, 0.1, 0.25 and 0.5ng/ml by HPLC analysis (Source: Generated during analysis, 2017)

4.10.1.3.2. Recovery

Recovery tests using Immunoaffinity columns (IACs) were performed in duplicate to determine the efficacy of the analytical method by spiking pasteurized milk samples with AFM1 standard solutions at the levels of (0.02, 0.05, 0.1µg/L) and submitting them to free extraction procedures as a result values of recoveries by the HPLC were 85.8, 85.8, 80.8% which were classed as valid as per [72] agreement which is between 70% and 110%. The quantification limit (LOQ) established was 0.02µg/l and the detection limit (LOD) was 0.01µg/l.

4.10.2. Bacteriological Examinations

4.10.2.1. Total Viable Bacterial Count (TVBC)

The TVBC of pasteurized milk samples was done as per the procedures given in the [73] using pour plate method. Plate count agar (Oxoid, UK) was used. This test was carried out to determine the content of microbial contamination of pasteurized milk. 11 milliliter of milk sample was serially diluted in 99 ml of Butterfield's phosphate-buffered dilution water (ratio of 1:10) up to five dilutions. Sterile duplicate glass petri dishes of 90mm were labeled according to the dilution index. One ml of the dilutions was aseptically withdrawn using a sterile measuring pipette and delivered into an opened and sterile petri dish and then closed. The same was done for a duplicate petri dish. This was repeated till all the dilutions were pipetted into their corresponding plates up to 10^{-5}. This was followed by pouring about 15-20 ml of standard plate count agar which had been autoclaved at 121 °C for 15 min, cooled and tempered in a water bath at 47 °C. The sample and the agar were gently mixed by alternate clock and anti-clockwise rotations and left to solidify on the bench for about 30 min. The plates were inverted and incubated at 32+/-1°C for 48 hr. After incubation, plates inoculated with sample dilution yielding between 25 and 250 colonies were counted. Colony counts were made using colony counter and the number of bacteria in milliliter milk was calculated by the formula given by [73].

4.10.2.2. Total Coliforms Count (TCC)

The Total Coliforms Count of pasteurized milk samples were done as per the procedures given in the [74].10ml of milk sample was added into sterile flask having 90 ml of peptone water. After

mixing, the sample was serially diluted up to 10^{-3} and 1 ml of inoculum was mixed thoroughly with molten 15–20 ml Violet Red Bile Agar (HiMedia, India) solution which was previously held in a water bath at 47 °C. Two plates were inoculated with each dilution. After thoroughly mixing, the plated sample is allowed to completely solidify and overlaid with about 4ml of VRBA then incubated at 30 °C for 24 hr. typical purplish red colonies were considered as coliform colonies and confirmed by fermentation of lactose in brilliant green lactose bile broth (BGB) . Colony counts were made using colony counter. In dishes which contain typical colonies the actual number in both plates of a dilution was counted as per the formula given by [75].

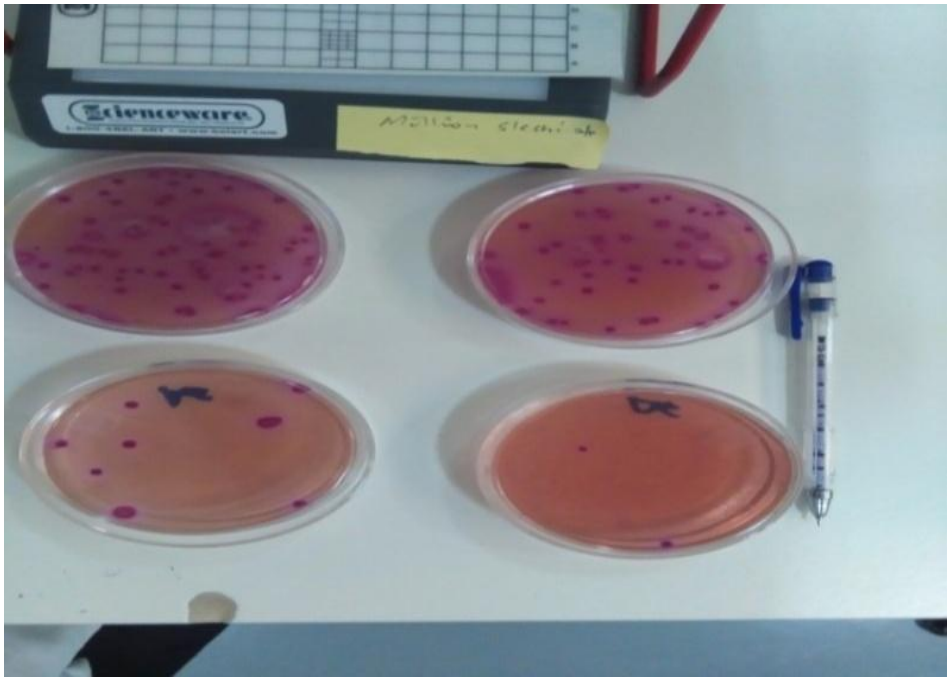


Figure 4.6: Coliforms on Violet Red Bile Agar (Source: Generated during analysis, 2017)

4.10.2.3. Testing for E. coli

Testing for E. coli in pasteurized milk samples was done as per the procedures given in the [73].Coliform colonies were examined for suspected E. coli. In order to increase chances of detecting E. coli up to six suspected E. coli colonies per plate were inoculated in to EC broth tubes and incubated 48h at 44°C. Then from each gassing EC broth tube loop full of suspension was streaked on L-EMB agar and those colonies with metallic sheen colored smooth sided were

transferred to PCA slants and incubated 18-24 h at 35°C for morphological and biochemical tests like for indole, methyl red, vogues proskaeuer and citrate (IMViC) reactions.

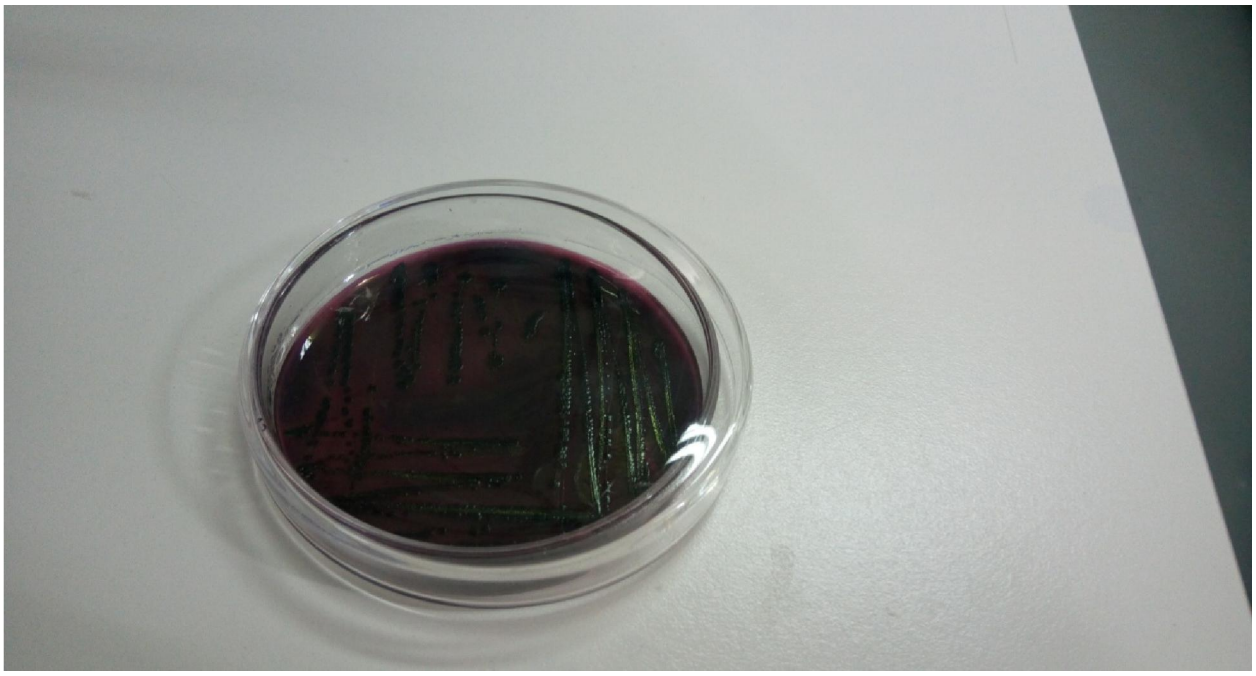


Figure 4.7: E. coli on L-EMB agar (Source: Generated during analysis, 2017)

4.10.3. Analytical Quality Assurance

At the beginning of the analysis/test, the column was washed by solvent; HPLC grade water for about ten minutes

Blank was always injected before each run and the peak was analyzed. Highest peak was expected at the beginning that shows peak of the solvent; again the column was washed (cleaned) with the solvent.

Pure standard is often injected before each run was undergone and the percentage of expected error was not exceeding 10%. In order to avoid this problem flushing or washing the column for a longer period, checking the storage condition of the standard and the working parameter of the parameters were considered.

Standard Reference Material was undergone once every three months or whenever big changes were observed on test method or instrument

ATCC organisms as a positive and negative control of the respective test, blank control media and air control were always run along with the test analysis during the bacteriological analysis.

4.11. Data Entry and analysis

Data obtained was entered and analyzed using Microsoft Excel and SPSS version 20 statistical software and considered 95% confidence interval.

4.12. Ethical consideration

Ethical approval for the study was granted by Ethical Clearance Committee of Addis Ababa University, Medical Laboratory Science Department and Addis Ababa Public Health Research and Emergency Management Core Process. All information obtained about any brand of the pasteurized milk produced and commercialized in Addis Ababa would be kept confidential. No name of companies would be displayed in publication and thesis work. Communication would be following ethical principle.

4.13. Dissemination of results

The results of this study were first presented to AAU department of Medical Laboratory Sciences and Addis Ababa Public Health Research and Emergency Management Core Process then to the research community through presenting at conferences and finally would be sent for publication on peer-reviewed scientific journals.

4.14. Operational definitions

- **Pasteurized Milk:** a milk or a fluid milk product which has been subjected to pasteurization which if retailed as such has been cooled without delay and has been packaged with minimum delay under conditions which minimize contamination. The product must give a negative phosphates test immediately after heat treatment.
- **Limit of quantification (LOQ):** is the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision.
- **Limit of detection (LOD):** is the minimum concentration of analyte that can be detected with acceptable certainty, though not quantifiable with acceptable precision
- **Coliforms**: are Gram-negative, rod-shaped facultative anaerobic bacteria. Identification criteria used are production of gas from glucose (and other sugars) and fermentation of lactose to acid and gas within 48 h at 35ºC
- **Fecal coliform**: rod-shaped bacteria that are non-spore forming, *Gram-Negative*, lactose-fermenting in 24 hours at 44.5 ° C, and which can grow with or without oxygen.
- ***E. coli*** : faecal coliform considered as being exclusively faecal in origin
- **Cfu**: A measure of viable cells in which a colony represents an aggregate of cells derived from a single progenitor cell.

5. Results

A total of Sixty five (65) 500 ml pre-packed pasteurized milk samples were analyzed in duplicate for presence of AFM1 and triplicate of total bacterial count, total coliform count and test for *E. coli* was performed.

5.1 Analysis of Aflatoxin M1

Analytical results showed that the occurrence of AFM1 contamination in pasteurized milk samples was high. Of the thirteen (13) brands investigated, Not Detectable (ND) AFM1 content was reported only from four of them, among which two batches of Brand **C** reported Not Detectable AFM1 content while only one batch of Brands **I, L**, and **M** reported Not Detectable AFM1content.

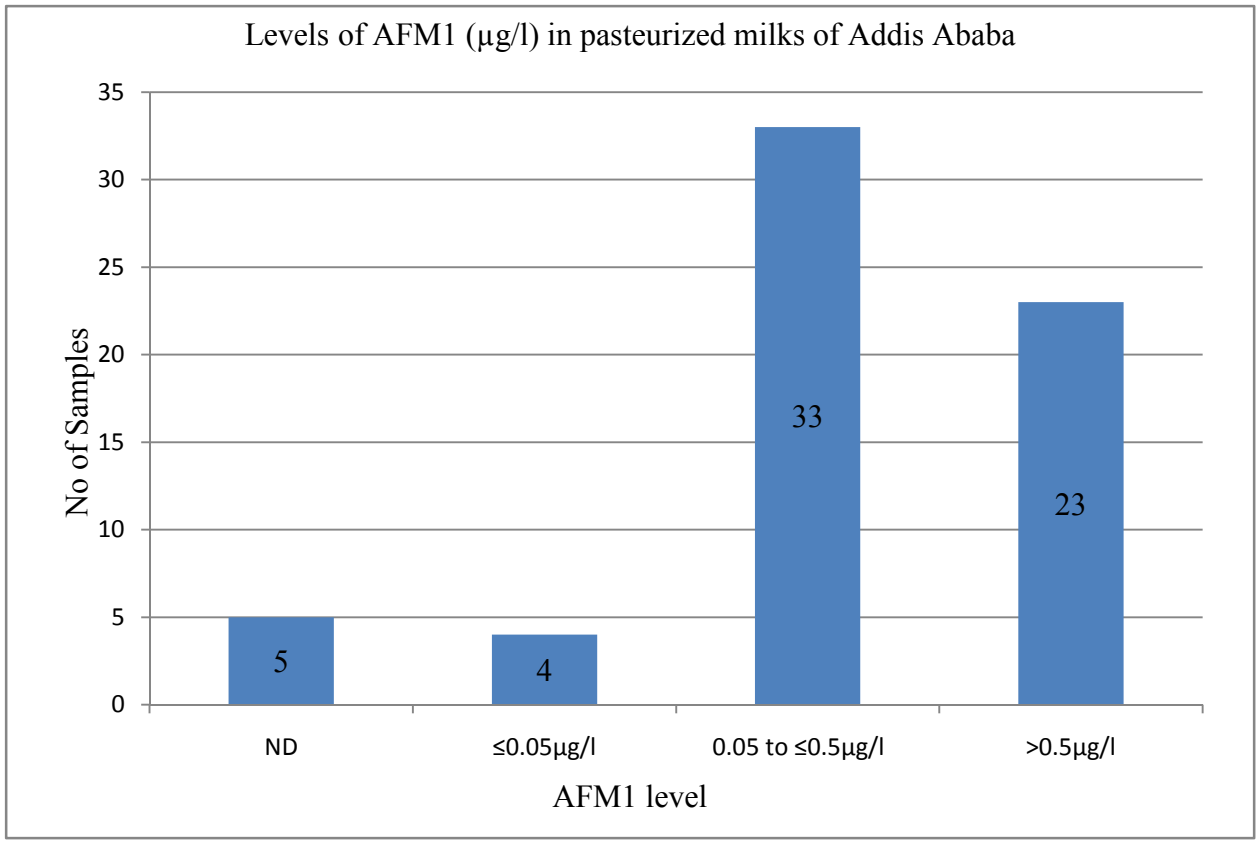
Table 5.1: Distribution of AFM1 concentration among different pasteurized milks in Addis Ababa from February 1 to December 15th 2017

Sample Code	AFM1 µg/l	Sample Code	AFM1 µg/l
A-1	0.607	**H-1**	0.677
A-2	0.590	**H-2**	0.143
A-3	0.632	**H-3**	0.190
A-4	0.524	**H-4**	0.392
A-5	0.389	**H-5**	0.023
	M= 0.548, SD= 0.098		**M= 0.285, SD= 0.256**
B-1	0.623	**I-1**	0.130
B-2	0.672	**I-2**	ND
B-3	0.709	**I-3**	0.148
B-4	0.696	**I-4**	0.186
B-5	0.568	**I-5**	0.180
	M= 0.654, SD= 0.058		**M= 0.131, SD= 0.071**
C-1	ND	**J-1**	1.216
C-2	0.622	**J-2**	0.194
C-3	0.141	**J-3**	0.843
C-4	ND	**J-4**	0.202
C-5	1.192	**J-5**	0.786
	M= 0.395, SD= 0.512		**M= 0.648, SD= 0.443**
D-1	0.450	**K-1**	0.143
D-2	1.398	**K-2**	0.127
D-3	0.206	**K-3**	0.024
D-4	0.985	**K-4**	0.092
D-5	0.414	**K-5**	0.048
	M= 0.691, SD= 0.489		**M= 0.087, SD= 0.051**
E-1	0.271	**L-1**	0.219
E-2	0.528	**L-2**	0.135
E-3	0.627	**L-3**	ND
E-4	0.224	**L-4**	0.137
E-5	0.202	**L-5**	0.168
	M= 0.370, SD= 0.194		**M= 0.134, SD= 0.077**
F-1	0.800	**M-1**	0.145
F-2	0.105	**M-2**	1.044
F-3	0.132	**M-3**	0.091
F-4	0.035	**M-4**	0.184
F-5	0.225	**M-5**	ND
	M= 0.259, SD= 0.310		**M= 0.295, SD= 0.424**
G-1	0.227		
G-2	0.952		
G-3	0.440		
G-4	0.630		
G-5	0.078		
	M= 0.465, SD= 0.343		
Mean = 0.382 ± 0.083 µg/l		**SD = 0.342 µg/l**	

A,B,C,D,E,F,G,H,I,J,K,L,M= Codes for brands ,1,2,3,4,5= Number of batches, ND= Not Detected, M= mean SD= Standard Deviation

AFM1 contamination was not detected in 5/65 (7.7%) and detected in 60/65 (92.3%) of pasteurized milk samples. The mean concentration of Aflatoxin M1 was 0.382 ± 0.083µg/l and the standard deviation was 0.342µg/l. The least AFM1 content was <0.02µg/l (ND) and the highest was 1.398µg/l.

Pasteurized milk samples of 4/60 (6.7%) had AFM1 levels less than 0.05µg/l, 33/60 (55%) had AFM1 levels between 0.05 and 0.5µg/l, and 23/60 (38.3%) of the samples had AFM1 levels above 0.5µg/l. *(figure 5.1).*



ND = Not Detected

Figure 5.1: Level of AFM1concentration among pasteurized milks (N=65) commercialized in Addis Ababa from February 1 to December 15th 2017

The mean concentration of Aflatoxin M1 per each brand was revealed in figure 5.2. The least mean AFM1 per brand was 0.087µg/l (Brand K) while the highest was 0.691µg/l (Brand D).

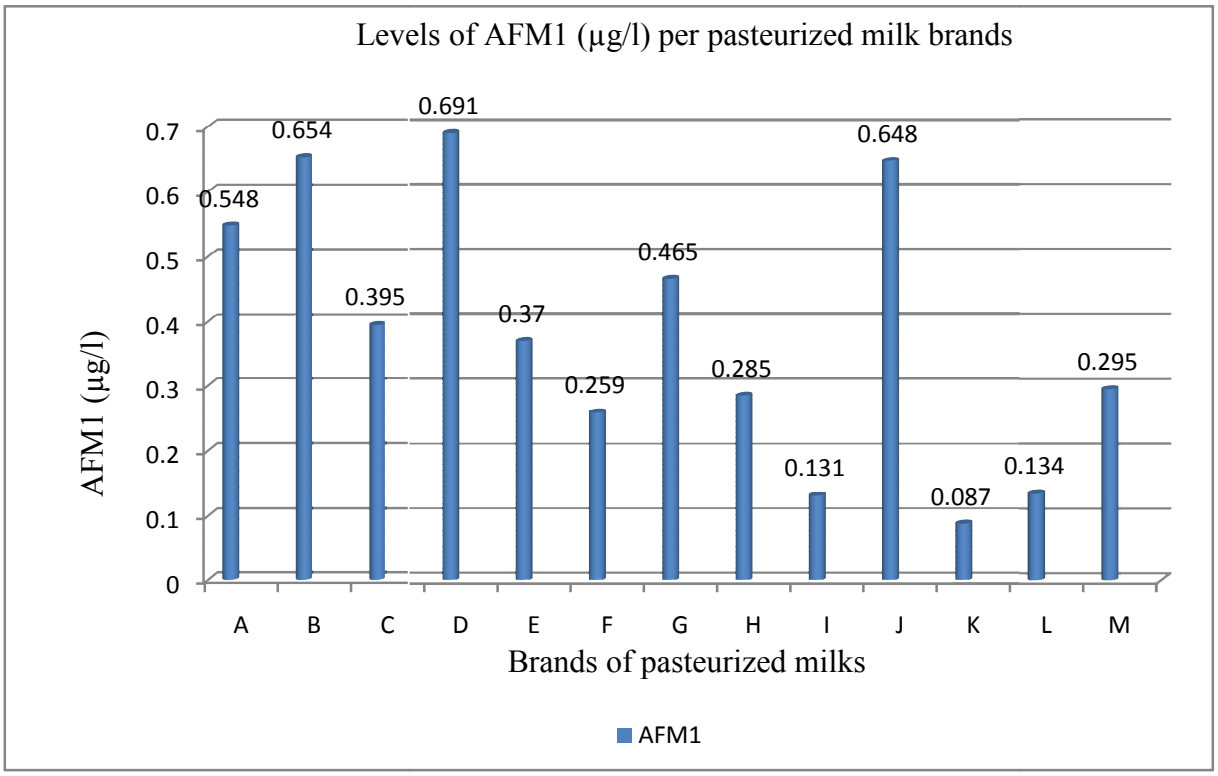


Figure 5.2: Average AFM1 (µg/l) concentration among different pasteurized milk brands in Addis Ababa, Ethiopia from February 1 to December 15th 2017

5.2 Total Viable Bacterial Count (TVBC):

The total viable bacterial count is the number of bacteria in a sample that can grow and form countable colonies on plate count agar after being held at 32 °C for 48 hours. The results of bacterial distribution of the pasteurized milk samples are presented in Table 5.2. The least TVBC in this study was <25cfu/ml and the highest was $2.7x10^6$cfu/ml.

Table 5.2: Distribution of TVBC among different pasteurized milks in Addis Ababa from February 1 to December 15th 2017

Sample Code	TVBC(Cfu/ml)	Sample Code	TVBC(Cfu/ml)
A-1	$1.8x10^5$	***H-1***	$2.2x10^6$
A-2	$2.0x10^5$	***H-2***	$3.1x10^3$
A-3	$1.8x10^5$	***H-3***	$5.5x10^5$
A-4	$2.0x10^5$	***H-4***	$1.1x10^5$
A-5	$2.0x10^4$	***H-5***	$1.4x10^3$
	M=$1.6x10^5$ SD=$7.7x10^4$		**M= $5.7x10^5$ SD= $9.4x10^5$**
B-1	$3.0x10^3$	***I-1***	$1.1x10^4$
B-2	$9.0x10^4$	***I-2***	$2.1x10^4$
B-3	$3.4x10^4$	***I-3***	$6.0x10^5$
B-4	$7.9x10^4$	***I-4***	$8.7x10^5$
B-5	$5.8x10^4$	***I-5***	$1.1x10^5$
	M= $5.3x10^4$ SD=$3.5x10^4$		**M= $3.2x10^5$ SD= $3.9x10^5$**
C-1	$6.2x10^3$	***J-1***	$2.2x10^5$
C-2	$1.4x10^3$	***J-2***	$1.9x10^5$
C-3	$2.4x10^4$	***J-3***	$9.8x10^4$
C-4	$2.3x10^4$	***J-4***	$8.5x10^4$
C-5	$1.8x10^4$	***J-5***	<25
	M= $1.5x10^4$ SD= $1.0x10^4$		**M= $1.2x10^5$ SD= $8.8x10^4$**
D-1	<25	***K-1***	$1.7x10^6$
D-2	$7.5x10^2$	***K-2***	$2.2x10^6$
D-3	$8.2x10^4$	***K-3***	$1.3x10^4$
D-4	$3.0x10^4$	***K-4***	$8.1x10^4$
D-5	$1.9x10^4$	***K-5***	$1.3x10^5$
	M= $2.6x10^4$ SD= $3.4x10^4$		**M= $8.2x10^5$ SD= $1.0x10^6$**
E-1	$2.7x10^6$	***L-1***	$1.0x10^5$
E-2	$2.3x10^6$	***L-2***	$9.4x10^4$
E-3	$1.1x10^5$	***L-3***	$2.0x10^3$
E-4	$2.8x10^4$	***L-4***	$2.8x10^4$
E-5	$2.1x10^4$	***L-5***	$2.1x10^4$
	M= $1.0x10^6$ SD=$1.3x10^6$		**M= $4.9x10^4$ SD= $4.5x10^4$**
F-1	$7.7x10^3$	***M-1***	$2.2x10^6$
F-2	$2.2x10^6$	***M-2***	$1.7x10^4$
F-3	$2.0x10^5$	***M-3***	$1.8x10^5$
F-4	$4.0x10^5$	***M-4***	$1.8x10^5$
F-5	$4.4x10^5$	***M-5***	$2.5x10^4$
	M= $6.5x10^5$ SD=$8.8x10^5$		**M= $5.2x10^5$ SD= $9.4x10^5$**
G-1	$8.8x10^5$		
G-2	$7.1x10^5$		
G-3	$4.1x10^5$		
G-4	$2.5x10^4$		
G-5	$7.0x10^5$		
	M= $5.5x10^5$ SD= $3.4x10^5$		
Mean= $3.8x10^5 \pm 1.7x10^5$ Cfu/ml		**SD= $6.8x10^5$ Cfu/ml**	

A,B,C,D,E,F,G,H,I,J,K,L,M=Codes for brands ,1,2,3,4,5= Number of batches, M= mean, SD= Standard Deviation
<25cfu/ml implies no growth of the microbe reported

The TVBC of the pasteurized milk samples in this study was ranged from $2.1x10^5$cfu/ml to $5.4x10^5$cfu/ml; and the average was $3.8x10^5 \pm 1.7x10^5$cfu/ml with standard deviation of $6.8x10^5$cfu/ml.

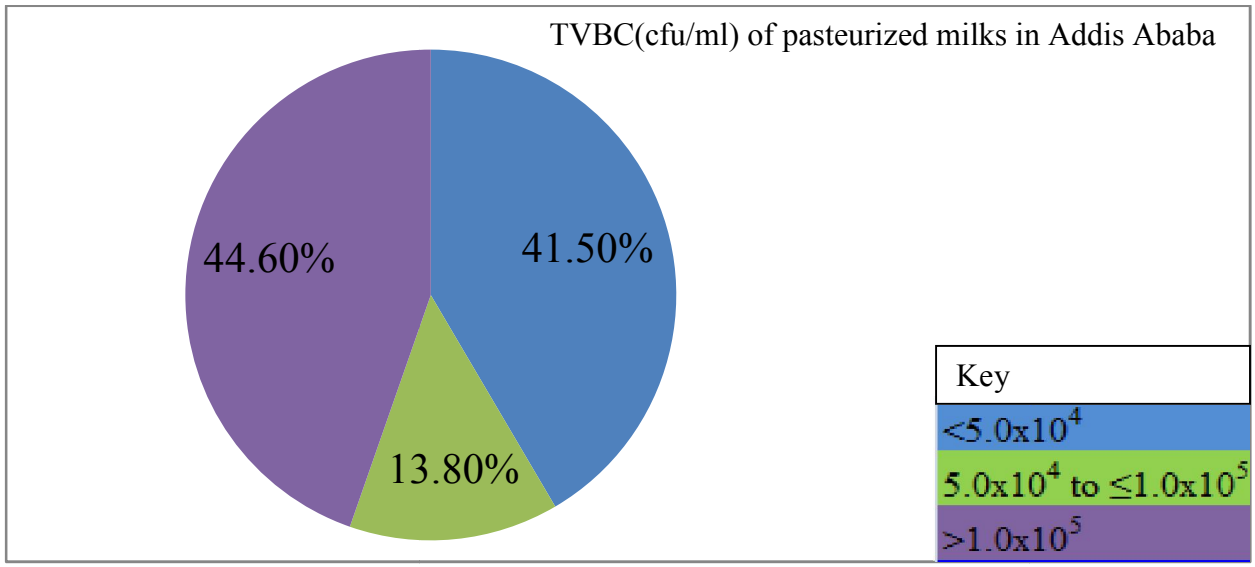


Figure 5.3: TVBC of different pasteurized milk samples (N=65) (cfu/ml) in Addis Ababa from February 1 to December 15th 2017.

The mean TVBC per each brand was revealed in figure 5.4. The least mean TVBC per brand was $1.5x10^4$cfu/ml (Brand C) while the highest was $1.0x10^6$cfu/ml (Brand E).

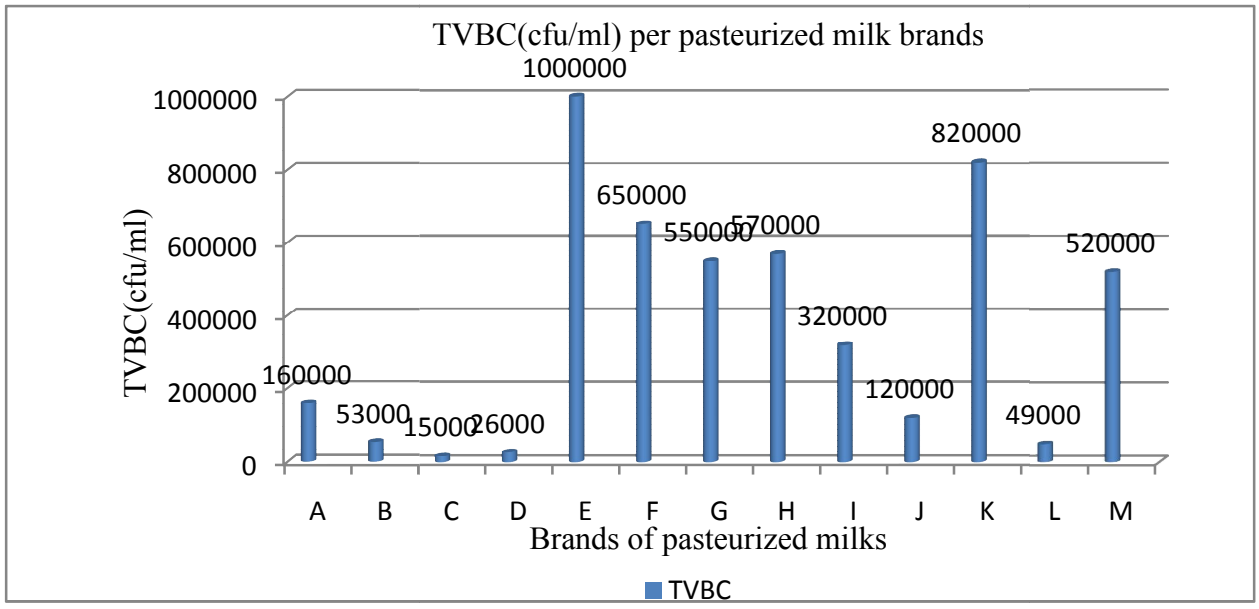


Figure 5.4: Average TVBC (cfu/m) among pasteurized milk brands in Addis Ababa, Ethiopia from February 1 to December 15th 2017

5.3 Total Coliform Count (TCC)/*E.coli*:

The TCC is the number of colonies in a sample that grow and form distinctive countable colonies on Violet Red Bile Agar after being held at 30°C for 24 hours. The results of coliform count distribution of the pasteurized milk samples are presented in Table 5.3. The least TCC in this study was <10cfu/ml and the highest was $1.7x10^3$cfu/ml.

Table 5.3: Distribution of TCC among different pasteurized milks in Addis Ababa from February 1 to December 15th 2017

Sample code	TCC(Cfu/ml)	Sample code	TCC(Cfu/ml)
A-1	$5.0x10^1$	***H-1***	<10
A-2	$2.0x10^2$	***H-2***	<10
A-3	$1.3x10^3$	***H-3***	$7.8x10^2$
A-4	$1.2x10^3$	***H-4***	<10
A-5	<10	***H-5***	$1.1x10^2$
	M= $5.5x10^2$ SD= $6.4x10^2$		**M= $1.8x10^2$ SD= $3.4x10^2$**
B-1	<10	***I-1***	$6.5x10^1$
B-2	$3.0x10^1$	***I-2***	<10
B-3	<10	***I-3***	<10
B-4	<10	***I-4***	$8.5x10^1$
B-5	<10	***I-5***	<10
	M= $6x10^0$ SD= $1.3x10^1$		**M= $3.0x10^1$ SD= $4.2x10^1$**
C-1	$2.5x10^1$	***J-1***	$1.1x10^2$
C-2	<10	***J-2***	$3.6x10^2$
C-3	<10	***J-3***	<10
C-4	$3.5x10^1$	***J-4***	$4.5x10^2$
C-5	<10	***J-5***	<10
	M= $1.2x10^1$ SD= $1.7x10^1$		**M= $1.8x10^2$ SD= $2.1x10^2$**
D-1	<10	***K-1***	<10
D-2	$4.0x10^1$	***K-2***	$1.6x10^3$
D-3	<10	***K-3***	$9.5x10^2$
D-4	$9.0x10^1$	***K-4***	$2.0x10^2$
D-5	$9.0x10^1$	***K-5***	$1.2x10^3$
	M= $4.4x10^1$ SD= $4.5x10^1$		**M= $7.9x10^2$ SD= $6.7x10^2$**
E-1	$1.2x10^3$	***L-1***	<10
E-2	$1.1x10^3$	***L-2***	<10
E-3	$3.0x10^1$	***L-3***	$8.3x10^2$
E-4	$7.0x10^1$	***L-4***	<10
E-5	$4.0x10^1$	***L-5***	$1.7x10^2$
	M= $4.9x10^2$ SD= $6.1x10^2$		**M= $2.0x10^2$ SD= $3.6x10^2$**
F-1	$7.0x10^1$	***M-1***	7.5x10
F-2	$3.7x10^2$	***M-2***	<10
F-3	<10	***M-3***	<10
F-4	<10	***M-4***	$1.5x10^2$
F-5	$4.1x10^2$	***M-5***	<10
	M= $1.7x10^2$ SD= $2.0x10^2$		**M= $4.5x10^1$ SD= $6.7x10^1$**
G-1	<10		
G-2	$1.7x10^3$		
G-3	$9.7x10^2$		
G-4	$1.9x10^2$		
G-5	$1.3x10^3$		
	M= $8.3x10^2$ SD= $7.2x10^2$		
Mean=$2.7x10^2$ ± $1.1x10^2$ cfu/ml		**SD= $4.6x10^2$ cfu/ml**	

A,B,C,D,E,F,G,H,I,J,K,L,M=Codes for brands ,1,2,3,4,5= Number of batches, M= mean, SD= Standard Deviation,,
<10cfu/ml implies no growth of the microbe reported.

The TCC of the pasteurized milks in this study was ranged from $1.6x10^2$cfu/ml to $3.8x10^2$cfu/ml and the average coliform count was $2.7x10^2 \pm 1.1x10^2$cfu/ml and the standard deviation was $4.6x10^2$cfu/ml.

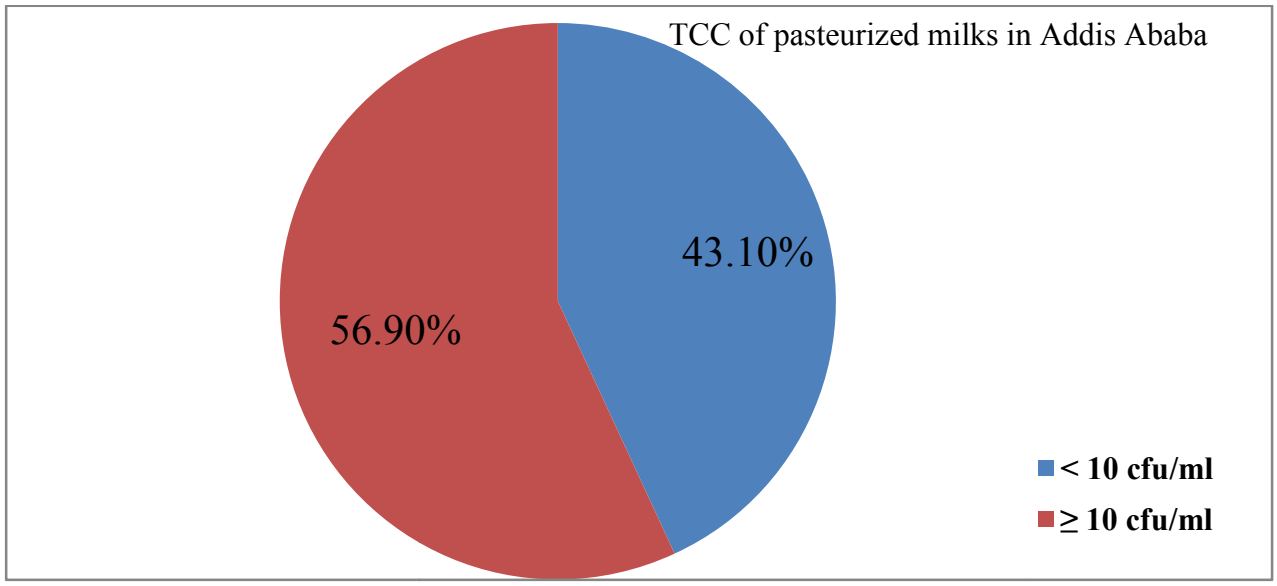


Figure 5.5: TCC among different pasteurized milks (N=65) in Addis Ababa from February 1 to December 15th 2017.

The mean TCC per each brand was revealed in figure 5.6. The least mean TCC per brand was $6x10^0$cfu/ml (Brand B) while the highest was $8.3x10^2$cfu/ml (Brand G).

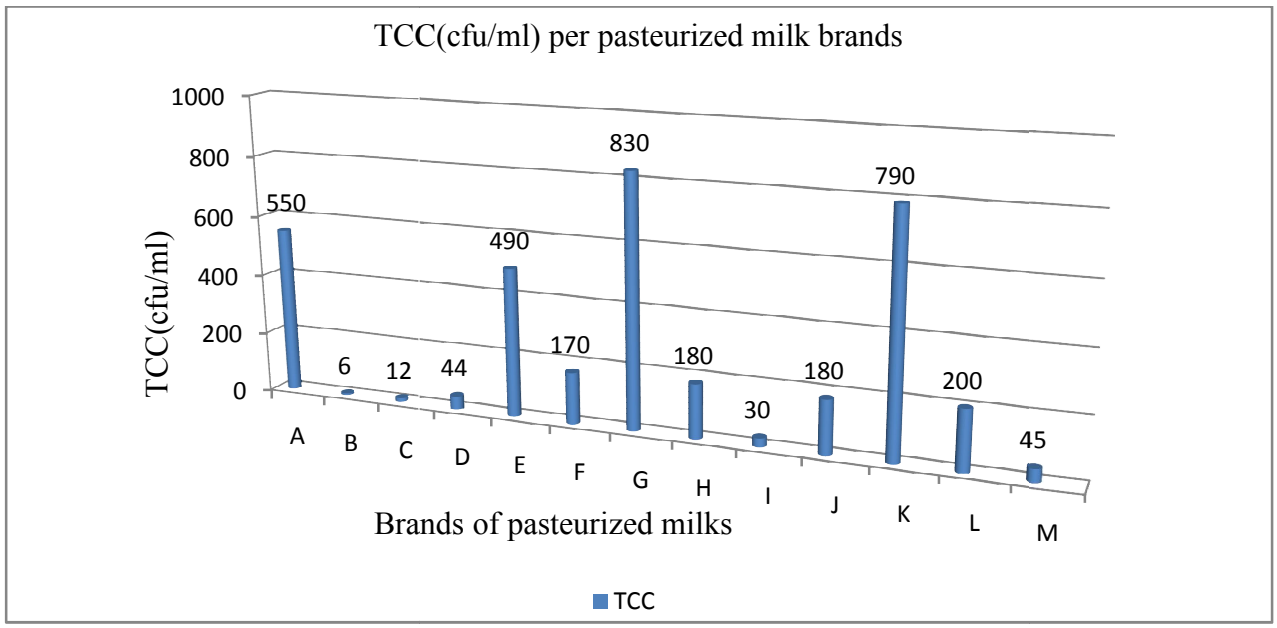


Figure 5.6: Average TCC among pasteurized milk brands in Addis Ababa, Ethiopia from February 1 to December 15th 2017

The presence of *Escherichia coli* (one of the member of Coliform bacteria) in pasteurized milk is a common indicator of fecal contamination. *E. coli* was isolated from 4 out of 65 (6.2%) pasteurized milk samples.

6. Discussion

The overall purpose of this study was to assess the level of AFM1 and bacterial quality in 500 ml pre-packed pasteurized milks commercialized in Addis Ababa and investigate whether the concentration of AFM1 and bacterial quality was within the acceptable level for consumption or not. This study revealed that pasteurized milks commercialized in Addis Ababa are highly contaminated with AFM1 and the bacteriological quality is substandard in terms of TVBC and TCC.

There was a high occurrence rate of AFM1 60/65 (92.3%) in the pasteurized milk samples. The majority, 56/60 (93.3%) of pasteurized milks exceeded the limit of 0.05µg/l set by the EU and Codex Alimentarius Commission while 23/60 (38.3 %) of the samples had AFM1 levels exceeding the maximum levels accepted by US FDA limit (0.5µg/l) and the highest concentration of AFM1 detected in this study was 1.398µg/l.

For mean AFM1 per brand, none of the brands met the maximum tolerable limit set by the EU (<0.05µg/l); while nine (69.2%) brands (Brands: C, E, F, G, H, I, K, L and M) met the standard set by the US FDA (<0.5 µg/l).

Though limited studies have been conducted on Aflatoxin M1 in pasteurized milk in Africa, In comparison, for example, studies from urban centers in Kenya have reported AFM1 levels up to 0.60 µg/l [38] and study conducted on the presence of AFM1 in pasteurized milk from Morocco have reported AFM1 level ranging from 0.001 to 0.117µg/l and a mean value of 0.0186µg/l [76], the study from Babol city, Mazandran province, Iran showed that the mean concentration of Aflatoxin M1 was 0.230µg/l, with a range of 0.178-0.254µg/l, which showed that the contamination level in all samples (100%) exceeded the EC regulations (0.05µg/l) [77]. However, the observed mean AFM1 concentration (0.382µg/l), with a range of 0.297µg/l – 0.465µg/l in this study was higher than which was reported in the African countries and Far East. This might be due to considerable feeding of cows with AFB1 contaminated feeds.

The result of AFM1 levels, derived in our study from pasteurized milk samples, was in agreement with Barbara S. *et al*., reported in Ethiopia. They reported that the levels of AFM1 in

samples of raw milk in the Great Addis Ababa milk shed was higher and about 93% of the milks exceeded the EU recommended limit [16].

Overall, the high levels of aflatoxin contamination of the pasteurized milk should be of concern for the dairy sector monitoring agencies, because Aflatoxin M1 can cause liver cancer and contribute to stunting [4].

In this study, 93.3% (56/60) of pasteurized milk samples exceeded the EU regulatory limit, most probably because of unusual and considerable using of contaminated feeds. Due to high contamination of samples, it should be mentioned that feeds for cows might be contaminated with AFB1. This fact appears to be a serious public health problem at the moment, since all age groups, including infants and children, consume milk worldwide; consequently, it is extremely important to maintain low level of AFB1 in the feeds of dairy animals. In order to achieve this, dairy cow feeds should be kept away from contamination as much as possible. Since the level of AFM1 was exceeded the maximum limits in 93.3% of the samples assessed in this study, imposing safety limits for AFM1 in pasteurized milk in Ethiopia seems to be an urgent need.

In the present study, the mean of TVBC in the pasteurized milks was $3.8x10^5 \pm 1.7x10^5$cfu/ml which was comparatively lower than similar studies conducted by Aberra A. in and around Addis Ababa, Ethiopia [71] and Ibtisam *et al*., in Khartoum (Sudan) [78], which reported as $2.1x10^6$cfu/ml and $7.9x10^{13}$cfu/ml respectively. Interestingly; two milk samples (D-1 and J-5) of our study showed no growth of microbes. However, it was higher than that recommended by USPHS (not exceeding 20,000 cfu/ml) [66], EAS (not exceeding 30,000 cfu/ml) [79], and ESA (not exceeding 100,000 cfu/ml) [70].

As per the bacteriological grading of ESA, 44.6% of the pasteurized milks should not have been for sell even though 41.5% and 13.8% of the milks were graded as very good and good qualities of the milks respectively.

For mean TVBC per brand, four brands (30.8%) (Brands: B, C, D and L) met the standard set by ESA (< 100,000cfu/ml) while nine brands (69.2%) (Brands: A, E, F, G, H, I, J, K and M) failed to met the standard.

Pasteurization has the advantage of reducing the transmission of disease producing bacteria, decreasing infant mortality, and improving the keeping quality of milk. Pasteurized milk presents little health hazard. Nevertheless, several food-borne diseases out breaks have been linked to pasteurized milk. There are several reasons for the occurrence of bacterial contamination in the pasteurized milk samples such as defect in pasteurization machinery, to survive even after pasteurization, and contamination in the post-pasteurized process due to poor processing and handling conditions and/or maintenance of substandard hygienic practices by working personnel [39].

In this study, the mean of TCC in the pasteurized milks was $2.7x10^2 \pm 1.1x10^2$cfu/ml which was comparatively lower than similar study conducted by Aberra A. in and around Addis Ababa, Ethiopia [71] and Ibtisam *et al*., in Khartoum (Sudan) [78], which reported as $3.1x10^5$cfu/ml and $4.33x10^{11}$cfu/ml respectively. However, it was higher than that recommended by USPHS, ESA and EAS (not exceeding 10cfu/ml) [66, 70, 79].

And above 56% (37/65) of the pasteurized milks were contaminated with coliforms for more than 10 cfu/ml; and *E. coli* was detected from 4 out of 65 (6.2%) pasteurized milk samples, which the ESA graded it as not for sell.

For mean TCC per brand, only one brand (7.7%) (Brand B) which revealed 6 cfu/ml, met the standard set by ESA (< 10cfu/ml) while the rest twelve brands (92.3%) (Brands: A, C, D, E, F, G, H, I, J, K, L, and M) failed to met.

Coliform bacteria are supposed to be absent in pasteurized milk as they can't survive the pasteurization temperature. Because of the defect in pasteurization process or post pasteurization contamination which includes contamination in packaging materials, defects in pipe lines, TCC may be detected in the pasteurized milk samples [68, 69].

7. Strength and Limitation of the study

7.1 Strength

Samples run were in duplicates for AFM1 Analysis and in triplicates for bacteriological analysis.

7.2 Limitation

Limited studies were available on similar sample type, making comparison difficult for our work and yet justifies the need for this study

8. Conclusion

According to the results obtained in Addis Ababa city, the occurrence and contamination levels of AFM1 in pasteurized milk seem to be a serious problem for public health, especially infants and children. The risk of Aflatoxin to human's health, especially liver cancer, has been proven by many researchers. The high cost of treatment is another point that cannot be overlooked. So, to produce high quality milk, it is essential to keep feeds free from contamination to AFB1.

The bacteriological quality of pasteurized milks marketed in Addis Ababa city at the time of this study does not satisfy the requirements set by the Ethiopian Standards Authority (ESA) for the quality of pasteurized milk. The TVBC and TCC in pasteurized milk should be of concern to the appropriate authorities; thus, the need for appropriate actions to be taken to assure consumer safety as well as ensure further processing of the pasteurized milk into value added products.

9. Recommendation

Therefore, it is recommended in order to ensure safety consumption of pasteurized milk, Aflatoxin B1 contaminated feeding for dairy cattle should be avoided. This seems to be the most practical way. Frequent analytical surveillance by food control agencies is highly recommended to control the incidence of mycotoxin contamination in Ethiopia especially in dairy products. Implementing a food control system, such as the HACCP system in the food industries, suggest an efficient means for limiting mycotoxin contamination in the Ethiopia's food supply.

Large scale studies should be conducted on much more milk samples and over more extended periods, in order to obtain data corresponding to various climates, humidity and temperature conditions. Additionally, the government authorities must converse and educate dairy farmers and dairy products consumers on how to store animal feeds and the potential health consequences of aflatoxins. And also:

- Awareness creation and training of milk handlers are needed at primary production levels and marketing to enhance quality and safety of the milk for consumers.
- Proper cleaning and sterilization of plant equipment and surfaces, and observing aseptic packaging is necessary to avoid milk post-pasteurization contamination.
- Processors need to increase severity of pasteurization regime based on microbial quality of milk.

References

[1]. JECFA (Joint FAO/WHO Expert Committee on Food Additives). Safety Evaluation of Certain Mycotoxins in Food: Aflatoxin M1. *WHO Food Additives Series* 2001; 47

[2]. Jay JM, Loessner J, Golden A. Modern Food Microbiology. New York: Springer Science and Business Media; 2005

[3]. Food and Agriculture Organization of the United Nations. Worldwide Regulations for Mycotoxins in Food and Feed in 2003. *Food and Nutrition Paper* 2004; 81

[4]. Gong Y, Cardwell K, Hounsa A, Egal S, Turner P, Hall A. Dietary aflatoxin exposure and impaired growth in young children from Benin and Togo. *Brit Med J* 2002; 325: 20-1.

[5]. Williams J, Philips D, Jolly P, Stiles J, Jolly C, Aggaewul D. Human aflatoxicosis in developing countries. *Am J Clin Nutr* 2004; 80: 1106-22

[6]. IARC. Monograph on the evaluation of carcinogenic risks in humans: Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. IARC 2002; 82: 171-274

[7]. Eaton D, Groopman J. The toxicology of Aflatoxin. Acadamic press. New York. 383-426

[8]. Anfossi L, Baggiani C, Giovannoli C, D'Arco G, Passini C, Giraudi G. Food Control 2012; 25: 125-130

[9]. Ehlers M. Municipal and Rural Sanitation. New Delhi: Mc Graw Hill Publisher; 1976

[10]. Godefay B, Molla B. Bacteriological quality of raw milk from four dairy farms and milk collection center in and around Addis Ababa. *Berl.Munich. Tierarzil wschr* 2000; 113:1-3

[11]. Karimi G. Study on the contamination of milk with aflatoxin in Tehran area using ELISA method. *J Pajoohesh Sazandeh* 1998; 40: 163-5

[12]. Kim E, Shon D, Ryu J, Park H, Hwang HJ, Kim Y. Occurrence of aflatoxin M1 in Korean dairy products determine by ELISA and HPLC. *Food Additive and Contaminants* 2000; 17: 59-64

[13]. Galvano F, Galofaro A, Ritieni M, Bognanno A, Angelis D, Galvano G. Survey of the occurrence of aflatoxin M1 in dairy products marketed in Italy. *Food Additive and Contaminants* 2001; 18: 644-646

[14]. Park D. Effect of processing on Aflatoxin. Advan. *Exp. Med. Biol* 2002; 504: 173-179.

[15]. Ghanem I, Orfi M. Aflatoxin M1 in raw, pasteurized and powdered milk available in the Syrian market. *Food Control* 2008; 20: 603-605.

[16]. Barbara S, Dawit G, Azage T, Jean H, Delia G. Aflatoxin contamination of milk and Dairy feeds in the greater Addis Ababa milk shed in Ethiopia. ILRI 2015

[17]. Tayganyilmaz M, Macdonell B, Corderocoma R, Gallagher M.Out breaks associated with unpasteurized milk and soft cheese; An overview of consumer safety. Food protection trends 2009; 29:211-222

[18]. WHO. Creating supportive environment for health Stories from the 3rd International conference on health promotion. Sundsvall Sweden 1996; 20-22

[19]. Mogossie A. Growth potential and inhibition of Bacillus cereus and staphylococcus aures during the souring of Ergo, a traditional Ethiopian Fermented milk; *Ethiopian Journal of Health Development* 1992; 6:23

[20]. Kazemi R, Mohammad M, Azizollahi M. Aflatoxin M1 Contamination in Milk and Milk Products in Iran. *Journal of Chemical Health Risks* 2013; 3: 13-20

[21]. Kiermier F, Meshaley R. Einfluss der molkereitechnischen behanlung der rohmilich auf des aflatoxin M1. Gehalt der daraus hergestellten produkte. Zeitschrift fur Lebensmittel untersuchung und forschung 1977; 164: 183-187

[22]. McKinney J, Cavanaugh G, Bell G, Bell A, Hoversland A, Nelson D, *et al*. Effects of ammoniation on aflatoxins in rations fed lactating cows. *Journal of American Oil Chemical Society* 1973; 50: 79-84

[23]. Stoloff L, Wood G, Carter G. Aflatoxin M1 in manufactured dairy products produced in the United States in 1979. *Journal of Dairy Science* 1981; 64: 2426-2430

[24]. Choudhary P, Sharma R, Borkartria V. Effect of chilling and heating on aflatoxin M1 content of contaminated Indian cow's milk. *Egyptian Journal of Dairy Science* 1998; 26: 223–229

[25]. Sinha K. Detoxification of mycotoxins and food safety. *New York: Marcel-Dekker* 1998;

[26]. Deveci O. Changes in the concentration of aflatoxin M1 during manufacture and storage of White Pickled cheese. *Food Control* 2007; 9: 1103-1107

[27]. Govaris A, Roussi V, Koidis A, Botsoglou N. Distribution and stability of aflatoxin M1 during processing, ripening and storage of Telemes cheese. *Food Additives and Contaminants* 2001; 5: 437-443

[28]. Grant D, Carlson F. Partitioning behavior of aflatoxin M1 in dairy products. Bulletin of the Environmental Contamination and Toxicology 1971; 6: 521-524

[29]. Brackett R. Marth E. Fate of aflatoxin M1 in parmesan and mozzarella cheese. *Journal of Food Protection* 1982; 45: 597-600

[30]. Kiermeier F. Aflatoxins residues in fluid milk. Pure Applied Microbiology 1973; 35: 271-273

[31]. Moreau C. Les mycotoxines dans les produits laitiers. Lait 1976; 55: 286-303

[32]. Alborzi A, Pourabbas B, Rashi M, Astanch B. Aflatoxin M1 contamination in pasteurized milk in Shiraz (south of Iran). *Food Control* 2006; 17: 582-4.

[33]. Oveisi MR, Jannat B, Sadeghi N, Hajimahmoodi M, Nikzad A. Presence of aflatoxin M1 in milk and infant milk product in Tehran. Iran. *Food Control* 2007; (In press)

[34]. Hossein T, Seyed MR, Mehran M. Detection of Aflatoxin M1 in raw and commercial pasteurized milk in Urmia, Iran. *Pak. J. Biol Sci. 2007*; 10 (22): 4103-07

[35]. Heshmati A, Milani M, Contamination of UHT milk by aflatoxin M1 in Iran. *Food Control* 2010; 21: 19 -22

[36]. Amer AA, Ibrahim M. Determination of aflatoxin M1 in raw milk and traditional cheeses retailed in Egyptian markets. *Journal of Toxicology and Environmental Health Sciences* 2010; 2: 50-53.

[37]. Kang'ethe EK, Lang'a KA. Aflatoxin B1 and M1 contamination of animal feeds and milk from urban centers in Kenya. *Afr Health Sci.* 2009; 9: 218–226

[38]. Tolle A. The microflora of the udder. Factors Influencing the Bacteriological Quality of Raw Milk. International Dairy Federation Bulletin 1980; Document 120: 4

[39]. ICMSF. Microorganisms in Foods. Microbial Ecology of Food Commodities. Blackie Academic and Professional, New York 1998; 6:521-576.

[40]. Teka G. Food hygiene principles and food borne disease control with special reference to Ethiopia. Faculty of Medicine, Department of Community Health, Addis Ababa University, Ethiopia 1997; 73-86.

[41]. FAO/WHO. Code of hygienic practice for milk and milk products. Joint FAO/WHO Food Standards Programme-Codex Committee on Food Hygiene, 26th Session, March 29-April 2, 2004, Washington, DC., USA.

[42]. North CE, Park WH. Standards for milk pasteurization. *Am. J. Hyg*. 1927; 7: 147-173

[43]. Hudson A, Wong T, Lake R. Pasteurization of dairy products: Times, temperatures and evidence for control of pathogens. Institute of Environmental Science and Research Limited, Christchurch Science Centre, New Zealand 2003; 1-55.

[44]. FDA. Grade A pasteurized milk ordinance. *Food and Drug Administration,* USA 2009; 1-26.

[45]. Okura H, Toft N, Nielsen SS. Occurrence of *Mycobacterium avium* subsp. paratuberculosis in milk at dairy cattle farms: A systematic review and meta-analysis. *Vet. Microbiol*. 2012; 157: 253-263

[46]. Stabel JR, Lambertz A. Efficacy of pasteurization conditions for the inactivation of *Mycobacterium avium* subsp. paratuberculosis in milk. *J. Food Protect.* 2004; 67: 2719-2726

[47]. Grant IR, Ball HJ, Neill SD, Rowe MT. Inactivation of *Mycobacterium paratuberculosis* in cow's milk at pasteurization temperatures. *Applied Environ. Microbiol*. 1996; 62: 631-636

[48]. Sharma SK, Sehgal N, Kumar A. Dry-reagent strips for testing milk pasteurization. *LWT-Food Sci. Technol.* 2003; 36: 567-571

[49]. Ludikhuyze L, Claeys W, Hendrickx M. Combined pressure-temperature inactivation of alkaline phosphatase in bovine milk: A kinetic study. *J. Food Sci.* 2000; 65: 155-160.

[50]. Vega-Warner AV, Wang CH, Smith DM, Ustunol Z. Milk alkaline phosphatase purification and production of polyclonal antibodies. *J. Food Sci.* 1999; 64: 601-605.

[51]. Singh V, Kaushal S, Tyagi A, Sharma P. Screening of bacteria responsible for the spoilage of milk. *J. Chem. Pharm. Res*. 2011; 3: 348-350

[52]. Aglawe PP, Wadatkar CM. Microbial examination of milk sample from nagpur region with reference to coliform. *Food Sci. Technol. Lett* 2012; 3: 24-26.

[53]. Okpalugo J, Ibrahim K, Izebe KS, Inyang US. Aspects of microbial quality of some milk products in Abuja Nigeria. *Trop. J. Pharm. Res* 2008; 7: 1169-1177

[54]. Anderson M, Hinds P, Hurditt S, Miller P, McGrowder D, Alexander-Lindo R. The microbial content of unexpired pasteurized milk from selected supermarkets in a developing country. *Asian Pac. J. Trop. Biomed* 2011; 1: 205-211

[55]. De Oliveira LP, Soares LS, Barros VC, Cirqueira MG. Study of *Staphylococcus aureus* in raw and pasteurized milk consumed in the Reconcavo area of the State of Bahia, Brazil. *J Food Process Technol*. 2011; 2

[56]. Al-Mazeedi HM, Gholoum FA, and Akbar BH. Microbiological status of raw and pasteurized milk in the state of Kuwait. *Int. J. Eng. Sci.* 2013; 3: 15-19

[57]. Vahedi M, Nasrolahei M, Sharif M, Mirabi AM. Bacteriological study of raw and unexpired pasteurized cow's milk collected at the dairy farms and super markets in Sari city in 2011. J. *Prev. Med. Hyg*. 2013; 54: 120-123

[58]. Silva R, Cruz AG, Faria JA, Moura MM, Carvalho LM,Water EH, *et al.* Pasteurized milk: Efficiency of pasteurization and its microbiological conditions in Brazil. Foodborne Pathog. Dis. 2010; 7: 217-219

[59]. Ryan CA, Nickels MK, Hargrett-Bean NT, Potter ME, Mayer L. Massive outbreak of antimicrobial-resistant Salmonellosis traced to pasteurized milk. *J. Am. Med. Assoc*. 1987; 258: 3269-3274

[60]. Upton P, Coia J. Outbreak of *E. coli* O157:H7 infection associated with pasteurized milk supply. Lancet, 1994; 344: 1015-1022.

[61]. Da Silva M, Hofer E, Tibana A. Incidence of *Listeria monocytogenes* in cheese produced in Rio de Janeiro, Brazil. *J. Food Prot*. 1998; 61: 354-356

[62]. Rysstad G, Kolstad J. Extended shelf life milk-advances in technology. *Int. J. Dairy Technol*. 2006; 59: 85-96

[63]. Cromie SJ. Microbiological aspects of extended shelf life products. *Aust. J. Dairy Technol.* 1991; 46: 101-104

[64]. Sourav K, Kamal K, Aftab U. Microbiological quality analysis of raw, pasteurized, UHT milk samples collected from different locations in Bangladesh. *Stamford Journal of Microbiology* 2014

[65]. Bangladesh Standards and Testing Institution. Bangladesh standard: Specification for pasteurized milk. Bangladesh Standards and Testing Institution 2002

[66]. Jay JM. Modern Food Microbiology. New Delhi: CBS Publishers and Distributor; 2003

[67]. Hossain TJ, Alam K, Sikdar D. Chemical and microbiological quality assessment of raw and processed liquid market milks of Bangladesh. *Res J. Dairy Sci* 2010; 4 (4): 28-34

[67]. Dey S, Karim M. Study on physicochemical and microbial quality of available raw, pasteurized and UHT milk during preservation. *Int. J. Sci. Invent* 2013; 2: 150-157

[69]. California Department of Food and Agriculture. New Coliform standard for milk sold to consumers. *California Department of Food and Agriculture* 2008

[70]. Ethiopian Standards Agency. Ethiopian Standard: Pasteurized liquid Milk-Specification. ES 3462:2009

[71]. Aberra A. Microbiological safety of pasteurized and raw milk from milk processing plants in and around Addis Ababa. Addis Ababa University, school of graduate studies 2010

[72]. European Commission (EC) REGULATION No 401/2006, "Laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs," *Official Journal of the European Union L* 2006; 70:12–34

[73]. Food and Drug Administration: **C**enter for **F**ood **S**afety **&** **A**pplied **N**utrition, *Bacteriological Analytical Manual*, 2001

[74]. Microbiology of food and animal feeding stuffs: Horizontal method for the enumeration of coliforms: Colony-count technique. ISO 4832:2006

[75]. Microbiology of food and animal feeding stuffs: General requirements and guidance for microbiological examinations. ISO 7218: 2015

[76]. Zinedine A, González-Osnaya L, Soriano JM, Moltó JC, Idrissi L, Mañes J. Presence of Aflatoxin M1 in pasteurized milk from Morocco. *Int J Food Microbiol.* 2007; 114:25-9

[77]. Sefidgar SA, Mirzae M, Assmar M, Naddaf SR. Aflatoxin M1 in Pasteurized milk in Babol city, Mazandaran province, Iran. *Iranian J Publ Health* 2011; 40:115-118

[78]. Ibtisam EM, Elmagli AA. Study on the Hygienic Quality of Pasteurized Milk in Khartoum State (Sudan). *Journal of Animal and Veterinary Sciences* 2006; 1(1): 12-17.

[79]. East African Standard: Pasteurized Milk- Specification. EAS 69:2006

Annex I: Laboratory test Procedure for AFM1 Analysis

Apparatus

HPLC system consisting of an auto sampler, pump, column, fluorescence detector, PC with chromatography software, HPLC column (ZORBAX SB-C8, 5µm, 4.6 × 150 mm, which was purchased from USA), Immunoaffinity column (Aflaclean M1 Select from LC Tech GmbH), Weighing balance, refrigerator, spatula, Beakers (100ml & 50ml), flask (250ml), measuring cylinder (50ml), funnel, sample preparation vials with screw cap and sealing ring, pipettes (1ml & 10ml), Whatman filter paper 4, Vaccum pump, lab tissues and other necessary laboratory glass wares and equipment were used for the analyses of the Aflatoxin M1at the Bless Agri food laboratory.

Chemicals and reagents for Aflatoxin analysis

Chemicals and reagents used for Aflatoxin M1 analyses were: HPLC grade water, acetonitrile, Aflatoxin standard. The Aflatoxin M1 standard was obtained from Sigma- Aldrich (Sigma Aldrich Chemie GmbH). The pure reference standard was stored in the dark at 4°C. Standard solutions of Aflatoxin M1 were prepared in acetonitrile and stored in the dark at 4°C for up to 6 months

- 100 ml milk sample was warmed at 37^0C in a water bath. The milk was gently stirred with magnetic stirrer to disperse the fat in the milk. The milk was centrifuged at 2000 x g for 15 minutes to separate fat from the residual milk. The thin fat layer is discarded.
- The milk was filtered through a Whatman No. 4 filter paper to remove any precipitations in the milk.
- The immune affinity column was adapted to room temperature and attached to syringe barrel then opened. A maximum of 50 ml filtered milk was applied on to the immune affinity column and let it pass through at slow steady flow rate of 2-3ml/min.
- To wash the column, the sample reservoir was rinsed with 20 ml water; Residue water was now removed by a gentle Nitrogen gas stream.
- 4 ml pure acetonitrile were applied on to the open column attached to a dry clean syringe barrel. Waited until the acetonitrile had reached the lower Luer exit of the column. The

column was immediately closed and incubated for 5 minutes, to efficiently break the analyte-antibody binding. After the column was re-opened, the eluate was collected in conical tube/beaker; by flushing air through the column residual eluate could be collected in the beaker.

- The eluate was evaporated to dryness using gentle stream of Nitrogen gas.
- The eluate was diluted to 1000µl (for 50µl injections) and measured by HPLC.

Calculation

Calculate Aflatoxin M_1 mass concentration of the test sample, using the following equation:

$$W_m = W_a \times (V_f / V_i) \times (1/ V_S)$$

Where:
W_m: the numerical value of Aflatoxin M_1 in the test sample in ng/ml (or µg /l);
W_a: the numerical value of the amount of Aflatoxin M_1 corresponding to area or height of the Aflatoxin M_1 peak of the test extract (ng); **X ng**
V_f: the numerical value of the final volume of re-dissolved eluate (µl); **1000 µl**
V_i: the numerical value of the volume of injected eluate (µl); **50 µl**
V_S: the numerical value of volume of prepared test portion passing through the column (ml): **50ml**
Express the results to 3 significant figures.

Annex II: Data collected from AFM1 Analysis

Sample No.	Trial 1	Trial 2	Average	Average (ng)X 0.4/ml	Sample No.	Trial 1	Trial 2	Average	Average (ng)X 0.4/ml
P-1	1.513	1.523	1.518	0.6072	***P-34***	1.552	1.596	1.574	0.6296
P-2	1.475	1.474	1.4745	0.5898	***P-35***	0.201	0.191	0.196	0.0784
P-3	1.592	1.569	1.5805	0.6322	***P-36***	1.702	1.685	1.6935	0.6774
P-4	1.309	1.312	1.3105	0.5242	***P-37***	0.384	0.329	0.3565	0.1426
P-5	0.794	1.150	0.972	0.3888	***P-38***	0.476	0.475	0.4755	0.1902
P-6	1.557	1.557	1.557	0.6228	***P-39***	1.026	0.933	0.9795	0.3918
P-7	1.659	1.702	1.6805	0.6722	***P-40***	0.051	0.063	0.057	0.0228
P-8	1.804	1.739	1.7715	0.7086	***P-41***	0.310	0.334	0.322	0.1288
P-9	1.771	1.707	1.739	0.6956	***P-42***	0.024	0.031	0.0275	0.0110=ND
P-10	1.422	1.416	1.419	0.5676	***P-43***	0.365	0.373	0.369	0.1476
P-11	0.022	0.025	0.0235	0.0094=ND	***P-44***	0.466	0.463	0.4645	0.1858
P-12	1.565	1.547	1.556	0.6224	***P-45***	0.462	0.436	0.449	0.1796
P-13	0.356	0.350	0.353	0.1412	***P-46***	3.077	3.002	3.0395	1.2158
P-14	0.046	0.045	0.0455	0.0182=ND	***P-47***	0.501	0.469	0.485	0.194
P-15	3.101	2.858	2.9795	1.1918	***P-48***	2.126	2.088	2.107	0.8428
P-16	1.260	1.238	1.249	0.4996	***P-49***	0.499	0.512	0.5055	0.2022
P-17	3.502	3.490	3.496	1.3984	***P-50***	1.991	1.938	1.9645	0.7858
P-18	0.536	0.496	0.516	0.2064	***P-51***	0.354	0.359	0.3565	0.1426
P-19	2.499	2.425	2.462	0.9848	***P-52***	0.315	0.322	0.3185	0.1274
P-20	1.038	1.030	1.034	0.4136	***P-53***	0.053	0.066	0.0595	0.0238
P-21	0.677	0.680	0.6785	0.2714	***P-54***	0.224	0.235	0.2295	0.0918
P-22	1.271	1.367	1.319	0.5276	***P-55***	0.118	0.121	0.1195	0.0478
P-23	1.580	1.553	1.5665	0.6266	***P-56***	0.554	0.541	0.5475	0.2190
P-24	0.581	0.539	0.56	0.2240	***P-57***	0.335	0.342	0.3385	0.1354
P-25	0.522	0.490	0.506	0.2024	***P-58***	0.043	0.046	0.0445	0.0178=ND
P-26	2.029	1.972	2.0005	0.8002	***P-59***	0.344	0.339	0.3415	0.1366
P-27	0.263	0.261	0.262	0.1048	***P-60***	0.416	0.425	0.4205	0.1682
P-28	0.329	0.333	0.331	0.1324	***P-61***	0.356	0.371	0.3635	0.1454
P-29	0.089	0.084	0.0865	0.0346	***P-62***	2.617	2.602	2.6095	1.0438
P-30	0.540	0.583	0.5615	0.2246	***P-63***	0.226	0.231	0.2285	0.0914
P-31	0.566	0.571	0.5685	0.2274	***P-64***	0.481	0.437	0.4590	0.1836
P-32	2.416	2.346	2.381	0.9524	***P-65***	0.034	0.036	0.0350	0.0140=ND
P-33	1.153	1.047	1.099	0.4396					

Annex III: Laboratory test Procedure for TVBC Analysis

Equipment and materials:- Petri dishes, glass or plastic, pipets with pipet aids or pipettors1, 5, and 10 ml, graduated in 0.1 ml units , dilution bottles, Erlenmeyer flask, borosilicate-resistant glass, rubber stoppers or plastic screw caps, circulating water bath(45 ± 1°C), Incubator(32 ± 1°C), Colony counter, refrigerator(0-5°C), refrigerator, freezer, thermometer(mercury) appropriate range, tally register, pipet and petri dish container, Dilution blanks,

Media

Plate count agar

Sample preparation

11ml of the sample was measured into a sterile 250ml Erlenmeyer flask; marked to indicate 100ml volume. Sterile 99 ml Butterfield's phosphate-buffered dilution water was added to 100ml mark. Dissolved and shacked thoroughly

Inoculation:

1ml of the milk homogenate of each dilution was pipetted into each of the appropriately marked duplicate dishes.

15-20ml of the molten PCA kept at 45±1 ^{0}C of water bath was poured into each duplicate Petri dishes.

Dilutions: 1:10, 1:100, 1:1000, etc

Dilution factor: 1 x 10^{1}, 1×10^{2}, 1×10^{3} etc

Homogenate was mixed by shaking then 1ml was pipetted into a tube (labeled 10^{-2}) containing 9ml of Butterfield's phosphate-buffered dilution water. Mixed carefully by aspirating 10 times with a pipette

From the 10^{-2} dilution, with the same pipette 1ml was transferred to the tube (labled10^{-3}) containing 9ml of the diluents, Mixed with a fresh pipette

The above procedure was repeated until the required numbers of dilutions were made.

Incubation

The dishes were inverted and incubated at 32±1^{o}C for 48±2 hr.

Computing and recording counts

When plates for all dilutions had no colonies, APC was reported as less than 25 colonies estimated count. Rounded by raising the second digit to the next highest number when the third digit was 6, 7, 8, or 9 and used zeros for each successive digit toward the right from the second digit and rounded down when the third digit was 1, 2, 3, or 4; When the third digit was 5, rounded up when the second digit was odd and rounded down when the second digit was even.

Plates with 25-250

$$N= \frac{\sum c}{V[n1+n2(0.1)]d}$$

Where: C = is the sum of colonies on all plates counted

V = is the volume applied to each plate

n_1= is the number of plates counted at first dilution.

n_2= is the number of plates counted at second dilution,

d = is the dilution from which first count was obtained.

N= is the average plate count.

Verification: If there was growth on the blank control and /or no growth on the positive control the test should be repeated with the corrected media

Expression of results: Expressed the result in cfu per ml

Annex IV: Data Collection Form of TVBC

Sample Code_____

Date of Sampling_____

Date analysis initiated_____ Date of test result report_____

Dilution	Aerobic plate count Plate 1	Aerobic plate count Plate 2
10^{-1}		
10^{-2}		
10^{-3}		
10^{-4}		
10^{-5}		
10^{-6}		
Blank test		
Positive control		
Air control		

Result Calculation: $\mathbf{N=\frac{\sum C}{V[n1+n2(0.1)]d}}$

C: _____ V: _____ $n_{1=}$_____$n_{2=}$_____d=_____N=_____ cfu/ ml

Where: C = is the sum of colonies on all plates counted

V = is the volume applied to each plate

n_1= is the number of plates counted at first dilution.

n_2= is the number of plates counted at second dilution,

d = is the dilution from which first count was obtained.

N= is the average plate count.

Annex V: Laboratory test Procedure for TCC Analysis

Equipments and Materials

Oven, autoclave, Incubator, Petri dishes of diameter 90mm to 100mm, Pipettes, Water bath, Colony-counting equipment, Test tubes, Durham tubes, Bottles or flasks, pH-meter, Loop

Media and reagents

Solid selective medium: Violet red bile lactose (VRBL) agar

Confirmation medium: Brilliant green lactose bile broth (BGB), EC broth, L-EMB, PCA

Tryptone broth, MR-VP broth, Koser's citrate, Kovacs' reagent, Gram Stains

Procedures

Inoculation and incubation

Two sterile Petri dishes were taken; using a sterile pipette, 1 ml of the test sample was transferred to each dish.

Into two sterile Petri dishes, using a sterile pipette, 1 ml of the first decimal dilution (10^{-1}) of the test sample was transferred to each dish. Into two other sterile Petri dishes using a fresh sterile pipette, 1 ml of the second decimal dilution (10^{-2}) of the test sample was transferred to each dish. The procedure described was repeated with the further dilutions, using a fresh sterile pipette for each dilution

About 15ml of the VRBL medium was poured, at 44^0C to 47^0C, into each Petri dish.

Carefully the inoculum was mixed with the medium and the mixture was allowed to solidify, with the Petri dishes standing on a cool horizontal surface.

Also a control plate was prepared with 15 ml of the medium for checking its sterility.

After complete solidification, about 4ml of the VRBL medium was poured, at 44^0C to 47^0C, onto the surface of the inoculated medium and allowed to solidify as described above.

The prepared dishes were inverted and incubated in the incubator set at 30^0C for 24 h ± 2 h.

Enumeration

After the specified period of incubation, Petri dishes with purplish red colonies (sometimes surrounded by a reddish zone of precipitated bile) were selected and using the colony-counting equipment the colonies were counted.

Confirmation

Five colonies of each were inoculated into tubes of brilliant green lactose bile broth (EC broth for E. coli). Incubated the tubes in the incubator set at 30^0C (44^0C for E. coli) for 24h +/- 2h. That showed gas formation in the Durham tube was considered as coliform colonies. The results were taken into account in the calculation

Expression of Results:

Using the following formula the number of colonies of Coliforms was calculated per ml of the milk.

a= b/A*C

Where,

a= number of colonies complying with identification or confirmation criteria

b= number of colonies complying with identification criteria among the inoculated colonies, A

A= number of colonies inoculated

C= the total number of presumed suspect colonies counted on the dish

$\sum a$= the sum of the colonies counted on the two dishes retained from two successive dilutions.

$$N= \frac{\sum a}{v[n1+(n2)0,1]d}$$

Confirmed test for *E. coli*

From each gassing EC broth tube loop full of suspension was streaked to L-EMB agar, one portion of plate must exhibit well-separated colonies and was incubated 18-24 h at 35°C.Plates were examined for suspicious *E. coli* colonies, i.e., dark centered and flat, with or without metallic sheen. 2 suspicious colonies from each L-EMB plate were transferred to PCA slants for morphological and biochemical tests. PCA slants were incubated 18-24 h at 35°C.

Gram stain was performed: All cultures were examined appearing as Gram-negative short rods or cocci.

The following biochemical activities were done:

1. Indole production. Tube of Tryptone broth was inoculated and incubated 24 ± 2 h at 35°C. Indole was tested by adding 0.2-0.3 ml of Kovacs' reagent. Appearance of distinct red color in upper layer was positive test.

2. Voges-Proskauer (VP)-reactive compounds. Tube of MR-VP broth was inoculated and incubated 48 ± 2 h at 35C. 1 ml to 13 x 100 mm tube was transferred. 0.6 ml a-naphthol solution and 0.2 ml 40% KOH were added, and shacked. A few crystals of creatine were added. Shacked and let stand 2 h. Test was positive if eosins pink color was develop.

3. Methyl red-reactive compounds. MR-VP tube was incubated additional 48 ± 2 h at 35°C after VP test. 5 drops to methyl red solution were added to each tube. Distinct red color was positive test. Yellow was negative reaction.

4. Citrate. Lightly tube of Koser's citrate broth was inoculated; detectable turbidity was avoided and incubated 96hr at 35°C. Development of distinct turbidity was positive reaction.

Interpretation: All cultures that were (a) appeard as Gram-negative non-spore forming rods or cocci, and (b) given IMViC patterns ++-- (biotype 1) or -+-- (biotype 2) were considered to be *E. coli*.

Annex VI: Data Collection Form of TCC

Sample Code_____

Date of Sampling_____

Date analysis initiated_____

Date of test result report_____

Dilution	Plate 1	Plate 2	Confirmation			Remark
			BGB	EC	L-EMB	
10^{-1}						
10^{-2}						
10^{-3}						
10^{-4}						
Positive Control						
Blank run						

Result calculation

a= b/A*C

Where,

a= number of colonies complying with identification or confirmation criteria

b- is number of colonies complying with identification criteria among the inoculated colonies, A

A-is number of colonies inoculated

C-is the total number of presumed suspect colonies counted on the dish

$\sum a$- is the sum of the colonies counted on the two dishes retained from two successive dilutions.

$$\mathbf{N=\frac{\sum a}{V[n1+n2(0.1)]d}}$$

$\sum a$= _____, v=_____, $n_{1=}$_____$n_{2=}$_____,d=_____, N=_____cfu/ml

N= <1/d where no colony grows on each dilution plates

Annex VII: Declaration

I, the undersigned candidate, declare that this thesis is my original work and has not been presented for a degree in this or any other university and all resources used for this thesis have been acknowledged.

Name of the student: Million Sleshi Weldemedhin

Signature: _____

Date: _____

Approval of the Advisors

Mr. Kassu Desta

Signature: _____

Date: _____

Mr. Samuel Kindie

Signature: _____

Date: _____