

ADDIS ABABA UNIVERSITY  
COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES  
CENTER FOR FOOD SCIENCE AND NUTRITION



Prevalence, antimicrobial resistance profiles and assessment of the risk factors associated with microbial quality and Shiga toxin-producing *Escherichia coli* (STEC) along the milk and cottage cheese value chains in Oromia, Ethiopia

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This Thesis is submitted to the Center for Food Science and Nutrition, College of Natural and Computational Sciences, Addis Ababa University; In Partial Fulfilment of the Requirements for Master of Science Degree in Food Science and Nutrition

June, 2021  
Addis Ababa, Ethiopia

## **Declaration**

I, the undersigned, declare that this Thesis entitled “**Prevalence, antimicrobial resistance profiles, and assessment of the risk factors associated with microbial quality and Shiga toxin producing *Escherichia coli* (STEC) along the milk and cottage cheese value chains in Oromia, Ethiopia**” has been carried out and written by me in the Center for Food Science and Nutrition, Addis Ababa University, Addis Ababa, under the supervision of **Dr. Ashagrie Zewdu** and **Dr. Tesfaye Sisay**. The information derived from the literature has been fully acknowledged in the text and list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

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**Date and place of submission: Center for Food Science and Nutrition**

**Addis Ababa University**

**June, 2021**

**Addis Ababa University**  
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## List of Abbreviations and Acronyms

CFU	Colony Forming Unit
CT-SMAC	Cefixime Tellurite-Sorbitol MacConkey
DAEC:	Diffusely Adherent <i>E. coli</i>
EAE	Entimin
EAEC:	Enteroggregative <i>E. coli</i>
EHEC :	Enterohemorrhagic <i>E. coli</i>
EIEC :	Enteroinvasive <i>E. coli</i>
EPEC:	Enteropathogenic <i>Escherichia coli</i>
EMB:	Eosin Methylene Blue
ETEC:	Enterotoxigenic <i>E. coli</i>
HACCP	Hazard Analysis Critical Control Point
HC:	Haemorrhagic Colitis
ISO	International organization for standardization
MPN	Most Probable Number
PCR:	Polymerase Chain Reaction
SPSS	Statistical Package for the Social Sciences
STEC:	Shiga-toxin producing <i>E. coli</i>
<i>Stx</i> :	Shiga toxin
TAE	Tris-acetic acid EDTA
VT:	Vero Toxin
VTEC	Vero toxin-producing <i>E. coli</i>

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## Abstract

Milk being a suitable medium for bacterial growth can serve as a source of bacterial pathogens. Pathogenic bacteria in milk pose a serious health threat to humans. Previous studies in Ethiopia lacked molecular confirmation of bacterial isolates, been small in terms of scope (i.e., failing to cover several high milk production areas), have not addressed all value-chains for milk and cottage cheese. Therefore, this study was designed to fill these gaps. aimed to assess the microbial quality, the prevalence of shiga-toxin producing *Escherichia coli* (STEC and *E. coli* O157:H7), antimicrobial resistance profiles, and associated risk factors in milk and cottage cheese sourced from the Oromia region of Ethiopia. First, a semi-structured questionnaire was administered to capture relevant information from identified respondents (producers, collectors, processors and retailers of milk and cottage cheese producers and retailers). Additionally, a total of 480 samples milk and cottage cheese were collected and analyzed for the presence of STEC and *E. coli* O157:H7. TCC and generic *E. coli* counts were completed utilizing 3M Petrifilm count plates, typical colonies were later confirmed through molecular methods. The antimicrobial resistance pattern of isolates was carried out using Kirby–Bauer method. The mean TBC and TCC of raw milk, pasteurized milk, and cottage cheese were (7.17, 5.53; 5.93, 3.36; 6.1, 1.73) log cfu/ml or g, respectively. The prevalence of generic *E. coli* was 53.1% (102/192), 35.9% (69/192) and 3.1% (3/48) in raw milk, pasteurized milk and cottage cheese samples, respectively. From the total samples, 12 % were positive for STEC, among this 3.7% were *E.coli* O157:H7. The highest prevalence of STEC and *E. coli* O157:H7 were found in raw milk samples 39 (20.31%) followed by pasteurized milk 17 (8.85%) and cottage cheese 2 (2.08%). Antimicrobial resistance analysis revealed that STEC and *E. coli* O157:H7 were highly sensitive to chloramphenicol (72.4%) and ciprofloxacin (75.8%); but resistant to ampicillin (56.8%), oxytetracycline (44.8%) and tetracycline (84.4%). The significant association of possible sources ( $P < 0.05$ ) with STEC positivity were cleanness of the drying cloth , hygienic condition of the cattle barn and collection center, temperature during transportation, time to transport pasteurized milk to retail shop and the quality of packaging materials for cottage cheese. Hence, there is a need to identify preventive measures and direct interventions for the production, transportation, storage and pasteurization of milk and cottage cheese.

**Keywords:** Milk, cottage cheese, Shiga toxin-producing *Escherichia coli* (STEC), antimicrobial resistance, risk factors

# 1. Introduction

## 1.1. Background of the study

Ethiopia has the largest livestock population in Africa estimated at about 59.9 million cattle, of which 11.83 million are milking cows currently producing 3.1 billion liters of milk (CSA, 2017). Ethiopia's dairy cattle population is distributed across most areas of the country, but the four regions with the highest number of milking cows are Oromia (44%), SNNP (22%), Amhara (17%) and Tigray (9%); from this the Oromia region is the leading milk producer in Ethiopia with 52 % of all milking cows in the country (CSA, 2019).

Oromia region is characterized by diversified agro-climatic zones, topography, agricultural potential and natural resources endowment (Mulat *et al.*, 2004). The region is contributing for 63% of the national volume of export of agriculture and share about 54% of grain production and 45% of livestock production from the country (CSA, 2014). West and East Shewa in the Oromia region have high market potential because of access to Addis Ababa (Gemedo *et al.*, 2018)

Ethiopia has a complex dairy value chain, with both formal and informal channels. Only 5% of the milk produced in Ethiopia is sold in commercial markets (LMD, 2012). Formal milk markets are particularly limited to peri-urban areas and Addis Ababa (Zegeye, 2003). The formal market appears to be expanding during the last decade with the private sector entering the dairy processing industry in Addis Ababa, DireDawa and Dessie towns (SNV, 2008). In Ethiopia, 2% of the national milk reached to the final consumers through formal value chain, whereas 98% of the milk is unprocessed and marketed through informal channel (Tekelyesus, 2015). Input suppliers, milk producers, milk processors and consumers involved the formal milk value chain. Milk provides a typical example with growing demand for milk value added products in Ethiopia, whereas local supply is limited (Beyene, *et al.*, 2015). In the informal market, milk may pass from producers to consumers directly or it may pass through two or more market agents. The informal system is characterized by no licensing requirement to operate, low cost of operations, high producer price compared to formal market and no regulation of operations, non-market oriented and most of the milk produced is retained for home consumption (Dehinenet, 2014).

Formal milk marketing of pasteurized milk and milk products accounts for fewer than 30% of total milk sales in Addis Ababa even though these products are hygienically prepared and

considered safe for human consumption (Tekliye, and Gizaw, 2017) However, inadequate or faulty pasteurization will not destroy all food borne pathogens (Pal *et al.*, 2012) and post-pasteurization contamination of milk can occur when pathogens are not adequately controlled in the food processing environment.

Foodborne diseases and food poisoning are a widespread and serious public health concern for individuals and countries of the modern world (Carbas and Coelho, 2013). Particularly in developing countries, the production of milk and various dairy products take place under unhygienic conditions and poor production practices (Bereda *et al.*,2012). Milk-borne pathogens cause human diseases ranging from gastrointestinal disturbances characterized by diarrhea and vomiting to others, generalized, and even life threatening foodborne illnesses, they are not only of public health importance but also economic importance (Quigley *et al.*, 2013).

Microbial assessments of bacterial load within milk is a frequently used procedure to measure the quality of milk. The Total plate count (TPC) and Total coliform count (TCC) methods are universal methods to estimate the total aerobic and coliform bacterial numbers present in raw milk (Fatine, 2012). With the procedures of the U.S. Food and Drug Administration (FDA 1978), the definition of a presumptive coliform is an organism that produces gas in lauryl sulfate tryptose broth in 48 hr at 35°C. A confirmed coliform is a presumptive coliform that produces gas in brilliant green lactose bile broth in 48 hr at 35°C. The coliform group includes *Escherichia coli*, *Citrobacter jreundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, and *Klebsiella pneumoniae*. There are a few strains of other species that ferment lactose and might be included in a coliform determination (Banwart, 2012).

*Escherichia coli* (*E. coli*) is a subgroup of the faecal coliform and it can be distinguished from most other coliforms by its ability to ferment lactose at 44°C in the fecal coliform test, and by its growth and color reaction on certain types of culture media. When cultured on an eosin methylene blue (EMB) plate, a positive result for *E. coli* is metallic green colonies on a dark purple medium. *Escherichia coli* has an incubation period of 12–72 hours with the optimal growth temperature being 37°C. Unlike the general coliform group, *E. coli* is almost exclusively of fecal origin and their presence is thus an effective confirmation of fecal contamination. Most *E. coli* strains harmless, but some can cause serious illness in humans. Infection symptoms and signs include bloody diarrhea, stomach cramps, vomiting and occasionally, fever. The bacteria can also cause pneumonia, other respiratory illnesses and urinary tract infections (Donnenberg, 2002; CDC, 2014). They are among many pathogenic microorganisms which can access to milk and other dairy products, and considered

as a reliable indicator of contamination by manure, soil and contaminated water (Ekici and Dümen, 2019).

*E. coli* is a normal inhabitant of the intestines of animals and humans; its recovery from food may be of public health concern due to the possible presence of enteropathogenic and/or toxigenic strains which lead to severe gastrointestinal disturbance (Quigley *et al.*, 2013; WHO, 2004). Among the pathogenic *E. coli* strains Shiga toxin *E. coli* (STEC) is the most important group of food-borne pathogens because a small number of people with STEC infection may develop kidney failure and anaemia (due to destruction of the red blood cells that carry oxygen around the body). This is called haemolytic uraemic syndrome (HUS) (FAO/WHO, 2018). *E. coli* O157:H7, which is considered as a subtype of STEC, is known to cause human diseases as food borne pathogen and is determined by production of virulence factors, also known as verocytotoxins or Shiga toxins (*Stx*). This Shiga toxin is produced in the colon and damages the tissue resulting in bloody and hemolytic uraemic syndrome that has become a serious health problem in various countries (Esparza *et al.*, 2011). Cattle are thought to be a reservoir for STEC and many foodborne diseases have been associated with the consumption of raw milk and dairy products (Dehkordi *et al.*, 2014).

It has been estimated that most of the microbial contamination of raw milk occurs during the time of collection, handling, processing and distribution (Diao *et al.*, 2010). However, currently there is a lack of data on pathogenic *E. coli* (STEC) confirmed through petrifilm by using molecular method and antimicrobial resistance test on milk and cottage cheese in the dairy supply chain of Oromia region. To take a measurement knowing the potential value chain was mandatory, so this work aimed to fill this gap. In the value chains like, producers, collectors, processors and retailer's quality and safety of both milk and cottage cheese were assessed.

## **1.2. Statement of the Problem**

The handling and safety of milk and milk products is a great concern around the world and particularly in developing countries. In addition there are challenges with continuous maintenance of the cold chain with limited knowledge on the hygienic handling of milk and milk products (Zezelem, 2010). In Ethiopia, the coliform counts of milk and cottage cheese samples reported by a number of workers (Zelalem *et al.*, 2005; Ashenafi, 2006; Binyam, 2008; Seifu *et al.*, 2013) revealed the seriousness of the problem. Moreover, there are limited research reports on the value chain analysis. Thus, study was conducted to bridge the information gap on the microbial quality of raw milk, pasteurized milk and cottage cheese across the value chain

in Oromia regional state.

In Ethiopia, research related to one of the Shiga toxin producing *E. coli* serotypes (*E. coli* O157:H7) has been mostly focused on slaughter houses and animal abattoirs (Bekele, *et al.*, 2014; Abdissa *et al.*, 2017; Abreham, *et al.*, 2019). There were just a few studies conducted to determine the prevalence of STEC and specifically STEC O157:H7 in cow raw milk in different areas of the country (Mersha, 2010; Ayano *et al.*, 2013; Disassa *et al.*, 2017; Zemenu, 2017; Bedasa *et al.*, 2018). However, just a single study on pasteurized milk (Bedasa *et al.*, 2018) has been conducted to determine the prevalence of *E. coli* O157:H7 on milk and its products in Ethiopia, and yet more is needed. The methods used for isolation and identification of *E. coli* O157:H7 were variable across studies. One of these studies reported using the standardised method established by (ISO, 2001) (Bedasa *et al.*, 2018). Two studies isolated *E. coli* by Enrichment in EC broth, transfer to EMB agar, and later transferred the colonies to sorbitol McConkey agar to identify putative *E. coli* O157:H7 isolates, which were then confirmed via agglutination test (Disassa *et al.*, 2017; Abunna *et al.*, 2018). Another study isolated colonies on McConkey agar and later performed confirmation via sugar fermentation tests (including sorbitol) and indole test (Adugna *et al.*, 2013). Finally, one study used a Biolog Identification System to characterise and confirm *E. coli* O157:H7 isolated using Biolog Universal Growth medium (Mekuria and Beyene, 2014), among these studies no one used molecular confirmation techniques for STEC isolates. Therefore, this study aims at isolation and molecularly characterization of STEC from raw milk, pasteurized milk, and cottage cheeses, and determining the prevalence of STEC and other *EC O157*. This study is different in its kind by using molecular technique, covering several high milk potential areas of Oromia regional state and by targeting all value chains of milk and cottage cheese in the study sites.

In Ethiopia, a number of studies have been done on the prevalence and antimicrobial resistance patterns of *E. coli* from various clinical sources (Gebre-Sellassie, 2007; Disassa *et al.*, 2017; Zemenu, 2017), but there is limited research on food samples, so this study fill this gap to some extent by testing antimicrobial resistance of the isolates obtained from raw milk pasteurized milk and cottage cheese in Oromia regional state.

### **1.3. Objective**

#### **1.3.1. General objective**

The study aims to determine the prevalence, antimicrobial resistance profiles and assessment of the risk factors associated with microbial safety and Shiga toxin-producing *Escherichia coli* (STEC) in milk and cottage cheese across the dairy value chain in Oromia regional state, Ethiopia

#### **1.3.2. Specific objective**

- To enumerate the total bacterial, total coliform and *E. coli* count in raw, pasteurized milk and cottage cheese samples collected across the dairy value chain in Oromia region.
- To determine the prevalence of Shiga toxin-producing *Escherichia coli* (STEC) in raw and pasteurized milk, and cottage cheeses samples in the region.
- To determine antimicrobial susceptibility profiles of STEC isolates in raw, pasteurized milks and cottage cheeses across the dairy value chain in Oromia region.
- To assess the risk factors associated in raw and pasteurized milk, and cottage cheeses samples across the dairy value chain in Oromia region.

## **2. Literature review**

### **2.1. Milk production in Ethiopia**

Although Ethiopia has the largest cattle population in Africa, at 52 million, and of these 10.5 million dairy cattle, the yearly milk production is very low with low per capital annual milk consumption (19 liters) which is by far below the African average (40 liters) as well as the world average (105 liters) (FAO, 2011). Referring data from CSA, (AGP, 2013) described that Ethiopian milk production has increased significantly since 2000 indicating that national milk production was 1.2 billion liters in 2000, 3.2 billion liters in 2007, and 3.3 billion liters in 2012. The production and market system of milk in Ethiopia face severe constraints along with complex milk value chain both in formal and informal market channels. Only 5% of the milk produced in Ethiopia is sold in commercial markets while the rest of 95% is consumed and processed at home (CSA, 2012). Dairy producers and the downstream actors in the milk value chains face many challenges in getting milk to market. For the most part, milk collection, chilling and transport are not well organized and there are few economies of scale. Transaction costs are high and up to 20-35% of milk is spoiled or otherwise lost. Poor genetic makeup, insufficient access to proper animal feed and poor management practices all contribute to the low productivity levels (Felleke *et al.*, 2010). Moreover, very high price of milk and milk products also the major challenge to purchase by middle and low-income consumers (AGP, 2013).

### **2.2. Dairy value chain actors**

The value chain describes the full range of activities which are required to bring a product or service from conception, through the different phases of production (involving a combination of physical transformation and the input of various producer services), delivery to final consumer, and final disposal after use (Gereffi, 2014)

Value chain actors refer to those who involved in overwhelming of particular agricultural commodities at various levels of input, production, processing and marketing activities. They directly participate in the value chain (Sango, 2014). Value chain influencer's influences the activities of the value chain by as long as affects the regulators frame works, policies, infrastructures and administrative conditions. It needs addition of value chains as it progresses from producers to consumers. Therefore, it transforms and adds value at each stages of the value chain (Tegegne *et al.*, 2013).

### **2.2.1. Milk producers or farmers**

Milk producers are dairy producers who are actively engaged in production and marketing of dairy products and they are major actors and have performed many functions starting from dairy cow selection, providing feedings, building shelter up to milking, processing the milk traditionally and selling it to the market. They sell the surplus milk produced to the local markets, either as liquid milk or in the form of butter or cheese (Betelhem, 2020).

### **2.2.2. Milk collectors /cooperatives**

Village collectors are the local traders who collect milks from smallholder farmers. They sell the outputs directly to the cooperatives, hotels and consumers in the district. Collecting the raw milk from milk producer farmers in the study area is the major functions of village traders. Most of milk produced is purchased from farmers by village traders (Betelhem, 2020). The dairy cooperatives collect milk from village collectors and its members. In the process of milk collection and bulking, lactometer test is applied to assure the quality of milk and it is one of the guarantees to keep the market since consumers are relied on. They add a value like change the raw milk in to yoghurt and butter (Abebaw and Haile, 2013). There are 180 cooperatives engaged in milk production and marketing operating in different parts of Ethiopia (Abebaw and Haile, 2013). By means of providing bulking and bargaining services, cooperatives may enhance market access and help farmers avoid the hazards associated with a perishable product with uncertain and variable demand dairy industries and retail (Betelhem, 2020).

### **2.2.3. Milk processing industries and retailers**

Processors are any processing action taken to milk either for the purpose of adding value or for safety purpose. Retailing is the distribution process in which all activities are involved in selling to the final consumers. They can collect raw milk from farmers or milk collectors and finally, they distribute to the final milk consumers. The scenario in cottage cheese, they buy the cottage cheese from cottage cheese producers (farmers) and add the cost and finally sell to the consumers at open market. Although farmers can produce cottage cheese and also act as retailers, they differentiated from the retailers by low cost of selling (Keba *et al.*, 2020).

### **2.3. Milk consumption behavior and marketing systems in Ethiopia**

Ethiopia has a low level of milk consumption compared to other countries in the region

(Kenya) = 90 lt/cap; Uganda = 50 lt/cap). Even though Ethiopia has the largest inventory of milk producing animals (cattle, sheep, goats and camels), per capita consumption of milk is low compared to Kenya with fewer livestock and Sudan. The national per capita consumption of milk and milk products is estimated at 17 kg (Ahmed *et al.*, 2009). The consumption of milk and milk products varies geographically between the highlands and the lowlands and the level of urbanization (Mugambi *et al.*, 2015). In the lowlands, all segment of the population consumes dairy products while in the highlands the major consumers primarily include children and some vulnerable groups such as the elderly and women (Anteneh, 2010).

As is common in other African countries (e.g., Kenya and Uganda), dairy products in Ethiopia are channeled to consumers through both formal and informal dairy marketing systems. Recently, however, private businesses have begun collecting, processing, packing and distributing milk and other dairy products. Still, the proportion of total production being marketed through the formal markets remains small (Kosgey *et al.*, 2011). In the formal system milk is collected at cooperative or private milk collection centers and transported to processing plants. In this system, there are some how milk quality tests (alcohol and clot-on-boiling tests and density) up on delivery, and therefore the quality of milk is fairly secured. Producers supplying milk in this system pay a due emphasis in the production, storage and transportation of milk if their milk has to be accepted (Yilma *et al.*, 2011). In Ethiopia, 95% of the national milk is marketed through informal channels and is unprocessed. The traditional processing and marketing of dairy products, especially traditional soured butter, dominate the Ethiopian dairy sector (Haile, 2009).

### **2.4. Risk factors for bacterial contamination in different value chain**

Milk collected from healthy cows typically has low microbial load and is deemed free of pathogenic microbial contamination (Muehlhoff *et al.*, 2013; Kamana *et al.*, 2017). Microbial contaminants are most commonly introduced into milk during the milking practice and/or at subsequent milk processing steps (Assefa *et al.*, 2019; Tassew and Seifu, 2011). Conditions for contamination of raw milk at different critical points are due to less hygienic practice in pre milking udder preparation, sub-optimal hygiene of milk handlers and poor sanitation practice associated with milking and storage equipment's (Garedew *et al.*, 2012). Inadequate food safety laws, weak regulatory systems, lack of financial resources to invest in safer equipment and processing and lack of education for food-handlers also another reason for high burden of food

born disease in developing countries (Lovell and Ford, 2012). These problems occur in countries like Ethiopia where there are a number of challenges in acquiring appropriate milk handling equipment and where there is limited access to clean water (Amentie, *et al.*, 2016; Yeserah *et al.*, 2020; Lijalem and Zereu, 2015). This compounding effect deteriorates the quality, safety, and quantity of milk produced in the country, ultimately jeopardizing food security, public health, and agriculture development (Gemechu, 2015; SNV, 2008).

Equipment used for milk handling, storage, and transportation has an effect on the safety and quality of milk and is a major source of microbial contamination (Ahmedsham, 2018; Kamana, 2017). Microbiological contamination can result from equipment surfaces, especially joints, open seams, and dents that are difficult to clean properly and can harbor microorganisms such as spore-forming bacteria and *Listeria monocytogenes*, and can lead to microbial persistence within milk processing facilities (Muluken *et al.*, 2020). Many collection centres, cooperatives, and processing plants implement quality control measures through two different quality tests: a lactometer reading and an alcohol test. The lactometer combined with a thermometer reading determines the specific gravity of the milk to make sure there is no adulteration. The alcohol test determines if the milk has undergone too much fermentation to undergo further heat treatment. Unfortunately, neither of these tests can determine the presence of bacterial pathogens of public health significance. Hence, prevalence of bacterial pathogens becomes worrying from time to time across value chains including dairy cooperative and unions (Blackmon, 2017).

Pasteurization of milk inactivates most pathogenic bacteria. However, inadequate or faulty pasteurization will not destroy all food borne pathogens (Pal *et al.*, 2012), and post-pasteurization contamination of milk can occur when pathogens are not adequately controlled in the food processing environment. The same is true in Ethiopia that significant number of pathogenic microorganisms was found in milk samples that have pasteurized (Tesfaye *et al.*, 2013). Hygienic 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 (Tassew and Seifu, 2011). In cases when milk was not adequately pasteurized or was re-contaminated post-pasteurization, the temperature abuse during transportation to retail locations or during storage can result in microbial growth that increases quality and safety concerns above the level of that at the processing facility.

## **2.5. Microbial properties of milk and cottage cheese**

Dairy production, processing and utilization in Ethiopia are mostly traditional. Apart from consumption of whole fresh milk, dairy products are widely used and are considered as traditional recipe in the form of Butter, Ayib (cottage cheese) and Ergo to name the few (Abebe Bereda, *et al.*, 2014). According to (Seifu *et al.*, 2012), in most households of Ethiopia no attempt is made to control the fermentation process of milk and products manufactured under traditional systems generally have poor qualities and do not meet the acceptable quality requirements set by various regulatory agencies

### **2.5.1. Milk**

Milk is used throughout the world as a human food at least one form or more like yoghurt and cheese. It is virtually a sterile fluid when secreted into alveoli of udder. However, beyond this stage of production, microbial contamination might generally occur from different sources (Tasci, 2011). Milk is largely made up of water, within which a wide range of nutrients including vitamins, proteins, fats and carbohydrates are suspended. These rich nutritional contents, the production and processing procedures in commercial milk production render it susceptible to contamination by a host of pathogenic microbes that could cause diseases in humans. Therefore, milk is known to be an efficient vehicle for transmission of disease causing agents to humans (Garedew *et al.*, 2012).

Ethiopia milk is produced and marketed to consumer without being pasteurized or subjected to and quality standard. About 98% of the annual milk produced by subsistence farmers who live in rural areas where dairy processing in the country is basically limited to smallholder level and hygienic qualities of products are generally poor (Muluye, 2016). Moreover, in the traditional practice the status of the cleanliness of the milkers, the udder of the cow, the milking environment and the milking equipment's could be the chief sources of the initial milk contamination (Haile *et al.*, 2012). The traditional milk equipment is reported to be often porous and therefore a reservoir for many organisms and difficult to clean All these reasons might increase the microbial load of milk produced in traditional practices of Ethiopia. (Bereda *et al.*, 2014)

### **2.5.2. Ayib (cottage cheese)**

Among dairy products, cheese or cheese-related products have been mostly contaminated by different microbes. According to (Sulaiman and Hsieh, 2017), between 1998 and 2014, several dairy outbreaks in the United States resulted from the consumption of raw milk or cheese contaminated with Shiga-like toxins producing *E. coli*. The presence of *E. coli* and total coliform bacteria refers to poor hygiene conditions. Coliforms also widely found in many cheeses (Brooks *et al.*, 2012). However, in contrast to the presence of these microbes in raw and pasteurized fluid milk, and even in some other cultured products (e.g. yogurt), the presence of coliforms in cheese may not necessarily be negative. Cheese product characteristics, including moisture content, pH, salt content, ripening conditions, age of product, and culture all influence potential levels of and roles for coliforms and other microorganisms in the final product (Wolfe *et al.*, 2014; Trmci *et al.*, 2016). A survey of raw milk cheeses by (Brooks *et al.*, 2012) found that 5 of 41 commercially available raw milk cheese samples had detectable coliforms (i.e., >10 cfu/g). In a similar study, (Trmci *et al.*, 2016) surveyed 273 raw and pasteurized cheeses from the U.S. and other countries and found that 75 of those samples were positive for coliforms in concentrations above 10 cfu/g. Finally, (Trmcic *et al.*, 2016) reported that pasteurization, pH, water activity, milk type (e.g., cow milk), and rind type were cheese factors that significantly associated with coliforms in cheese. They also report that water activity is significantly associated with the final concentration of coliforms in cheese

In Ethiopia the safety of cheese with respect to food borne diseases is a great concern where the consumption of cottage cheese (Ayib) is typically manufactured in small dairy farms under poor hygienic conditions (Bereda *et al.*, 2014; Gemechu and Tola, 2017). Generally countries produced soft cheeses including Ethiopian cottage cheese due to poor keeping quality and hygienic conditions practiced during production, processing and handling lead to public health risk to the consumer (Mostert and Jooste, 2002).

### **2.6. Indicator microorganisms in milk and dairy products**

Indicator organisms indicate sanitary quality of foods and water. Coliforms can be found in the aquatic environment, in soil and on vegetation and they are universally present in large numbers in the feces of warm-blooded animals (Banwart, 2012). Coliforms themselves do not normally the presence of serious illness and they are easy to culture. Their presence is used to indicate other pathogenic organisms of fecal origin. Such pathogens include disease-causing bacteria, viruses, or protozoa and many multicellular parasites (Banwart, 2012).

A large number of people are suffering from diseases around the world which are directly caused by various food pathogens such as *Escherichia coli* (*E. coli*), *Campylobacter jejuni* (*C. jejuni*), *Listeria monocytogenes* (*L. monocytogenes*), *Shigella* spp and *Salmonella* spp which are mostly found in dairy product (Zhao, 2014). There are approximately 1.4 million cases of illness annually, resulting in 1000 deaths (Jasson, *et al.*, 2010). For instance, a study conducted in Mali found an increased risk of food-related intoxication characterized by diarrhea or vomiting in children consuming milk products (Millogo *et al.*, 2010) Similarly, (Darapheak *et al.*, 2013) showed an increased risk of diarrhea in children consuming milk in Cambodia. The consumption of raw milk and its derivatives is common in Ethiopia which is not safe from consumer health point of view as it may lead to the transmission of various diseases. Prior to the discovery and widespread adoption of pasteurization for instance, raw milk and its products were responsible for serious bacterial infections such as diphtheria, scarlet fever and tuberculosis (Sykora, 2003). In addition to hygiene concern lack of refrigeration facilities or continuous cooling system at the household level in developing countries of tropical regions, with high ambient temperature implies that raw milk will easily be spoiled during storage and transportation (Tassew and Seifu, 2011).

### **2.6.1. Coliform and *Escherichia coli* (*E. coli*)**

Coliform bacteria are Gram-negative, aerobic and facultatively anaerobic, rod-shaped bacteria that ferment lactose to produce acid. They produce  $\beta$ -galactosidase within 48 hours at 35 °C (Clesceri *et al.*, 1998). Among the genera *Escherichia*, *Klebsiella*, *Enterobacter*, *Serratia*, and *Citrobacter* (collectively called the coliforms) and *Proteus*, some are opportunistic pathogens responsible for a wide range of infections (usually not foodborne infections), but many species are members of the normal intestinal flora (Flannigan, 2016). Coliforms are almost always found in raw milk but with good methods of production number of coliforms can be kept very low (Elmoslemany *et al.*, 2010). The presence of these organisms in milk and milk products is an indication of unsanitary production and/or improper handling of either milk or milk utensils (Salman and Hamad, 2011). (Montel *et al.*, 2014) showed that in addition to faecal contamination, other factors such as milking wet udders, inadequate cooling of milk and udder infection are the main sources of coliform in bulk milk. (College of Agric and life Sciences, 2001) asserted that coliforms are associated with faecal and environmental contamination. Bulk milk Coliform bacteria are used as indicator of hygienic condition during handling and processing of milk and milk products (College of Agric and life Sciences, 2001).

*Escherichia coli* are considered a commensal of the large intestines of warm-blooded animals,

including man. The majority of *E. coli* rods do not constitute a serious health hazard, but some serotypes can cause food poisoning and alimentary intoxications. Some strains of *E. coli* are associated with diseases and can be subdivided into several pathological groups. *Escherichia coli* are serotyped based on the combination of O, H, and K antigens, although generally only the O and H types are listed, for example, *E. coli* O157:H7 (Italia *et al.*, 2012).

A wide variety of food has been implicated as a vehicle of *E. coli* infection including milk. *Escherichia coli* is an inhabitant of the intestinal tract of animals and humans (Wetzel, 2005). Six different groups of pathogenic *E. coli* strains exist that harbour various virulence factors which enable them to cause diarrheal disease; enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), and Diffusely Adherent *E. coli* (DAEC) (Croxen and Finlay, 2010).

## **2.7. Shiga toxin producing *E. coli* (STEC)/*E. coli* O157:H7**

STEC are a group of bacteria that produce one or more Shiga toxin (*Stx*). *Stx* is alternatively known as verotoxin or VT, and the STEC as verotoxigenic *E. coli* or VTEC. The STEC are of concern in human medicine because they cause food- or water-borne bloody diarrhea (also called hemorrhagic colitis (HC), and in some patients a more serious disease, the hemolytic uremic syndrome (HUS). The HUS is characterized by acute renal failure, thrombocytopenia and microangiopathic hemolytic anemia and is more common in children less than 5 years of age and the elderly (Lim *et al.*, 2010; Frank *et al.*, 2011). STEC was described for the first time in Canada by the end of the 1970s by (Gyles, 2007). All serotype O157:H7 have the same morphology. They are Gram negative, facultative anaerobic bacteria that belong to the Enterobacteriaceae family and the *Escherichia* genus (Xia, 2010; Farrokh *et al.*, 2012). So far over 400 STEC serotypes were identified, yet only a subset of these has been associated with diarrheal illnesses (Wang *et al.*, 2003; DebRoy *et al.*, 2011). Among the various serotypes *E. coli* O157:H7 is the most common and extremely pathogenic serotype indicated in diarrheal outbreaks worldwide (Bettelheim and Goldwater, 2014). Predominantly cattle are implicated as major reservoirs of STEC (Bolton *et al.*, 2009; Croxen *et al.*, 2013).

In east Africa, isolation of the pathogen has been reported in Tanzania, Kenya and Ethiopia. *E.coli* O157:H7 has been isolated from beef, mutton and chevron in Ethiopia at a prevalence of 8, 2.5 and 2%, respectively. In Ethiopia a contamination frequency of *E. coli* O157:H7 reported by (Zemenu, 2017) was 3.27% from raw milk and milking equipment samples collected from different dairy farms in Bishoftu town. In addition, an isolation frequency of 12% of the pathogen was reported from raw milk sample collected from open markets in Bishoftu town by (Bedasa *et al.*, 2018).

#### **2.7.1.1. Epidemiology of Shiga toxin producing *E. coli* (STEC) and *Escherichia coli* O157:H7**

It has been recognized for a number of years that STEC strains causing human disease belong to a very broad range of O: H serotypes (Chizhikov *et al.*,2001). Serotype O157:H7 was the first STEC type to be linked to outbreaks of HC and HUS (Chizhikov *et al.*.,2001,) In the United States,HUS occurs sporadically as well as in outbreaks half of STEC O157:H7 cases (43.4%) required hospitalization. The mortality rate of STEC infection in the US was low and death cases occurred exclusively in older age group (over 80 years) (CDC, 2011). During 2006 to 2010, the incidence of STEC infection in EU countries was on an increasing trend from 2006 to 2009and remained to stable in 2010. The 2011 outbreak of STEC O104:H4 in Europe was the most significant one with higher rate of HUS caused by STEC non-O157:H7 and a major proportion of middle-aged people being infected (CDC, 2011). Ireland reported the highest incidence rate, 4.41 per 100,000 population, followed by Sweden and Denmark, whose incidences were 3.58and 3.22 per 100,000 population respectively (Lim Esther *et al.*, 2011). The incidence of disease and systemic effects on the other hand by several virulence factors such as Shiga toxins (*Stx 1* and *Stx 2*), intimin (*eaeA*) and enterohemolysin (*hlyA*) (El-Seisy *et al.*, 2011). Besides *Stx1* or *Stx2*, and additional factors that may play a role in colonization (in either the animal or human host) or other steps in the pathogenic process. The clinical manifestation of acute phase associated with this organism is named hemorrhagiccolitis in humans. Thus, the symptoms characteristic to this disease are watery and/or bloody diarrhea, fever, nausea, severe abdominal cramping and vomiting (Callaway *et al.*, 2009; Walker *et al.*, 2012).

Furthermore, from the point of ingestion, the incubation period of the infection ranges from 8 hours to 16 days, but the typical incubation period is three to four days and the illness usually lasts 5-10 days (Robinson and McKillip, 2010). Additionally, life-threatening complications on some victims, particularly the very young, the elderly and those who are immunosuppressed or have debilitating illnesses, may develop haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (Karmali, 2009; Martorelli *et al.*, 2017). Thus, haemolytic uraemic syndrome is characterized by three features, acute renal failure, haemolytic anemia (reduction in the number of red blood cells) and thrombocytopenia (a drop in the number of blood platelets), sometimes preceded by a bloody diarrhea (Yeni *et al.*, 2016) and occurs in up to 15% of hemorrhagic colitis victims and can lead to permanent loss of kidney function (FDA, 2012). In addition, thrombotic thrombocytopenic purpura is a less common complication which is largely confined to adults. It is related to HUS but causes less kidney damage and includes fever and neurological symptoms resulting from blood clots in the brain (Chekabab *et al.*, 2013; Yeni *et al.*, 2016). Moreover, in clinical cases the mortality rate varies with the syndrome in humans. Thus, hemorrhagic colitis alone is usually self-limiting, although deaths can occur. The case fatality rate of the infection associated with HUS is estimated to be 1-10% and 50% in children and elderly, respectively. In European surveillance, the case fatality rate in all reported EHEC O157: An H7 infection was < 0.5% (CFSPH, 2016). STEC O157:H7 has been isolated from beef, mutton and chevon in Ethiopia at a prevalence of 8, 2.5 and 2%, respectively (Hiko *et al.*, 2008) as well as goat and sheep faeces (4.7%), skin swabs (8.7%), carcass before washing (8.1%), carcass after washing (8.7%) and water samples (4.2%) (Mersha *et al.*, 2010)

### **2.7.1.2. Diagnosis of STEC**

Infection with this agent is associated with a broad spectrum of illness ranging from mild diarrhea and hemorrhagic colitis to the potentially fatal haemolytic uraemic syndrome (HUS). Thus, these clinical symptoms are used as one diagnosis technique (Rahal *et al.*, 2012). Besides, clinical cases can be diagnosed by finding the organisms in faecal samples, food and environmental samples may also be tested to determine the source of the infection (CFSPH, 2016). Therefore, the conventional samples are diarrheic faeces in animals, predictable food item in both animal and human food (Elhadidy *et al.*, 2015). Moreover, detection of *E. coli* O157:H7 is based on phenotypic differences from most other serotypes, its inability to ferment sorbitol on Sorbitol MacConkey (SMAC) agar and absence of B-glucuronidase activity in most

strains. A presumptive diagnosis of the strain from these tests must then be confirmed serologically by *E. coli* O157:H7 latex agglutination test, by which the kit is commercially available (Yeni *et al.*, 2016). Also, immunoassays and polymerase chain reaction technology have led to the more rapid detection of this *E. coli* strain in stools. Thus, techniques included in this category are PCR and DNA-based techniques, immunomagnetic separation and enzyme-linked immunosorbent assays (ELISAs)(Bavaro, 2009). Also, molecular based techniques are distinctly advantageous because of their sensitivity, selectivity and their rapid results. However, molecular-based methods are appreciably more expensive than traditional plating techniques and are also more novel and unfamiliar. Therefore, the integration of molecular-based approaches into quality control procedures depends on the overall needs and resources of the food processing plant (Robinson and McKillip, 2010).

### 2.7.1.3. **Prevention and control of STEC**

Effective surveillance and epidemiological investigation systems, together with food safety control measures at all stages of the food chain are the basis for prevention and control for foodborne diseases like STEC infection. In Hong Kong, there are established disease surveillance and epidemiological investigation systems on human STEC infection, food surveillance programme and food incidents surveillance (Thorpe, 2004). There are also various statutory requirements and health promotional initiatives in safeguarding and promoting food safety along all stages of food chain (WHO, 2019)

In human, since the infection primarily occurs via faecal-oral route, the preventive measures include food hygiene measures like proper cooking of foods, consumption of pasteurized milk, washing fruits and vegetables especially those to be eaten raw and drinking chlorine treated water and personal hygiene measures like washing hands after toilet visits (Kiranmayi *et al.*, 2010). Moreover, the prevention of infection requires control measures at all stages of the food chain from agricultural production on the farm to processing, manufacturing and preparation of foods in both commercial establishments and the domestic environment (WHO, 2010). Thus, the hazard analysis critical control point system continues to be the most effective means for systematically developing food safety protocols that can reduce the risk of *E. coli* O157:H7 infections. Therefore, an important component of hazard analysis critical control point application in animal production at farms is reducing the carriage of the pathogen in animals. Furthermore, the two approaches that have the potential for prevention are competitive exclusion and vaccination (Karmali *et al.*, 2010)

### **2.7.1.3. Public health and Economic importance of STEC**

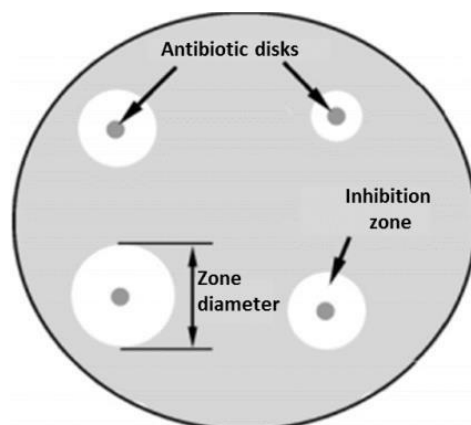
Shiga toxin producing *Escherichia coli* is an emerging public health concern in most countries of the world. (Kiranmayi *et al.*, 2010). The most common serotype of shiga toxin producing *E. coli* *E. coli* O157:H7 is estimated to cause 2.8 million acute illnesses each year (Majowicz *et al.*, 2014). Meanwhile, an estimated 73,480 illnesses due to *E. coli* O157: H7 infection occur each year in the United States, leading to an estimated 2,168 hospitalizations and 61 deaths annually (Rangel *et al.*, 2005; Scallan *et al.*, 2011) resulting in an economic burden of 607 million US dollars (Scharff, 2012), including 370 million US dollars for premature deaths, 30 million US dollars for medical care and 5 million US dollars in lost productivity (Frenzen *et al.*, 2005). In addition, patients who develop HUS often require prolonged hospitalization, dialysis and long term follow up, which are expensive in all directions (CFSPH, 2016). Furthermore, the infection costs billions of US dollars as a result of recalls, destroyed food, control measures and lost demand associated to loss of consumer confidence (Frenzen *et al.*, 2005).

### **2.7.1.4. Treatment of STEC**

Treating *E. coli* O157 infection with antimicrobial agents are associated with an increased risk of severe sequel such as HUS (Rahal *et al.*, 2012; Goldwater and Bettelheim, 2012) and may exacerbate the patient's condition by increasing either the release of preformed Shiga toxins (*Stx*) upon cell lysis. However, early administration using some antimicrobials is effective (Nassar *et al.*, 2013). Moreover, in an epidemiology study conducted by the Centers for Disease Control and Prevention, patients treated with antibiotics for STEC enteritis had a higher risk of developing HUS. Additionally, antibiotics promote Shiga toxin production by enhancing the replication and expression of Shiga toxin genes that are encoded within a chromosomally integrated lambdoid prophage genome. Shiga toxin induction also promotes phage-mediated lysis of the pathogen cell envelope, allowing for the release and dissemination of Shiga toxin into the environment (Sperandio and Nguyen, 2012). Therefore, treatment of hemorrhagic colitis is supportive and may include fluids and a bland diet (Xia, 2010). Thus, antibiotics are controversial and are usually avoided, they do not seem to reduce symptoms, prevent complications or decrease shedding, and as they may increase the risk of HUS (CFSPH, 2016).

### 2.7.1.5. Antimicrobial resistance

Conventional antimicrobial susceptibility testing methods require that pathogens are first isolated from the samples. In separate assays, isolated microorganisms are then exposed to various concentrations of antimicrobial agents under specified growth conditions, and the ability of these antimicrobs to inhibit growth is determined (figure 1). Test measured and the result is interpreted in accordance with criteria provided by (CLIS, 2012). Mueller-Hinton agar is frequently used in this antibiotic susceptibility test.



**Figure 1.** Kirby - Bauer antibiotic sensitivity tests Source: (CLIS, 2012)

Antimicrobial resistance in *E. coli* has been reported worldwide and increasing rates of resistance among *E. coli* is a growing concern in both developed and developing countries. Higher resistance of *E. coli* isolates has been reported this day for various antimicrobial agents. The descending resistance level of streptomycin (98.25%), tetracycline (98.09%), sulfonamides (90.31%), gentamycin (79.68%), chloramphenicol (73.8%), ampicillin (71.11%), trimethoprim (62.22%) ciprofloxacin (60.31%) and lowest resistance for nitrofurantoin (23.96%) was shown for *E. coli* isolates in Iran (Shahrani *et al.*, 2014).

In Ethiopia, the research done by (Disassa *et al.*, 2017) and (Zemenu, 2017) from raw cow milk samples in and around Asosa and Bishoftu towns of Ethiopia, respectively showed the presence of  $\geq 50\%$  resistance of *E. coli* O157:H7 against tetracycline, streptomycin, kanamycin, cefoxitin and chloramphenicol. On the other hand, 14.4% resistance isolates to drugs of ampicillin, vancomycin, streptomycin and tetracycline have been reported from milk and milk product samples collected from the open market and supermarket sources in Bishoftu town (Bedasa *et al.*, 2018).

## **2.8. Methods of enumeration, detection and identification of Shiga toxin producing *E. coli* (STEC)**

### **2.8.1. Conventional Methods**

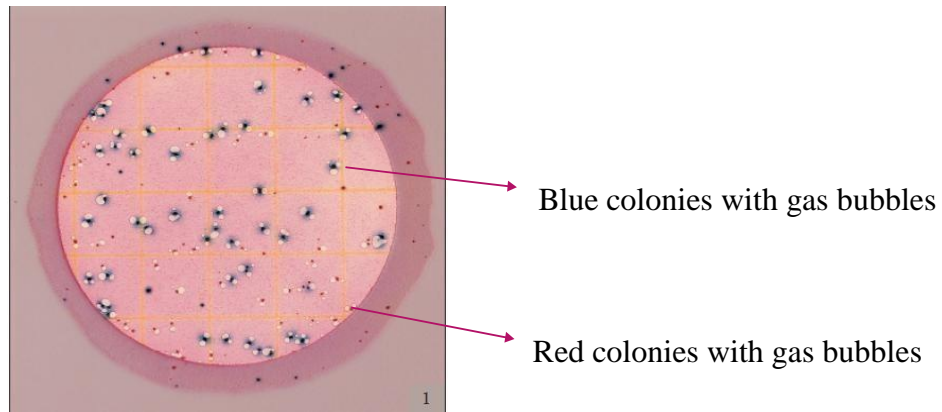
Traditional culture methods are used for the enumeration of coliform/ *E. coli* and for the detection of STEC. Standardized methods (e.g., ISO methods) are usually considered as the reference methods for official controls (Jasson *et al.*, 2010). Enumeration of the coliform and *E. Coli* present in a sample is normally performed by colony count method or the most probable number (MPN) method. The colony count method is based on culturing dilutions of sample suspensions in the interior or on the surface of an agar layer in a Petri dish. Individual microorganisms or small groups of microorganisms will grow to form individual colonies that can be counted visually (ISO, 4832). The MPN method calculates the number of viable microorganisms in a sample by preparing decimal dilutions of the sample, and transferring subsamples of 3 serial dilutions to 9 or 15 tubes containing liquid culture medium, to carry out the method on 3 or 5 tubes, respectively. The tubes are incubated, and those that show growth (turbidity) are counted. Taking into account the dilution factor, the final result is compared to a standard MPN table, which will indicate the MPN of bacteria in the product (Blodgett, 2010).

An International Organization for Standardization (ISO) method is available for the detection of *E. coli* O157:H7 in food and animal feedstuffs (ISO, 2001). This method is based on an enrichment procedure, followed by a separation and concentration step, and then an isolation step on selective chromogenic media. It was shown to be sensitive, with a detection limit of 1–2 CFU/25 g, as well as effective and applicable to the examination of dairy products (Voitoux *et al.*, 2002). Methods for standardization of non-O157:H7 STEC are in the process of being developed. One of the disadvantages with culture-based methods is that cells can enter a dormancy state where they become non-culturable (viable but non-culturable); (Dinu and Bach, 2011). This can lead to an underestimation of numbers or a failure to isolate a viable culture, although the cells may still retain pathogenicity, or be recoverable to a viable cell state. Recovery of cells may be compromised by selective or chromogenic media and non-culturable viable count should be considered in using these media. STEC O157:H7 can usually be easily distinguished from by their inability to ferment sorbitol within 24 hours on sorbitol-containing agar isolation media.

To isolate O157:H7 STEC should be plated onto a selective and differential medium such as sorbitol-MacConkey agar (SMAC), cefixime tellurite-sorbitol MacConkey agar (CT-SMAC), or CHROMagar O157:H7 (Church *et al.*, 2007). After incubation for 16-24 hours at 37°C (99°F), the plate should be examined for possible O157:H7 colonies, which are colorless on SMAC or CT-SMAC and are mauve or pink on CHROMagar O157:H7. Both CT-SMAC and CHROMagar O157:H7 are more selective than SMAC, which increases the sensitivity of culture for detection of O157:H7 STEC (Church *et al.*, 2007).

### **2.8.2. Rapid petrifilm test method**

Dry plate culturing (e.g., 3M Petrifilm or similar) is yet another widely used means of assessing microbiological quality of a wide range of foods for coliforms, *E. coli* aerobic mesophilic bacteria, psychrotrophs, and staphylococci. This system consists of multiple layers of plastic film encasing a dehydrated disk of the appropriate medium. Typically, a single sheet of plastic is aseptically peeled back and 1 mL of inoculum is used to rehydrate the medium while the film is reapplied and pressed flat. In some types of rehydratable films, gas production (e.g., from Enterobacteriaceae) may be detected as bubbles in the film following incubation. Dry media culture plates have been applied in settings ranging from predicting shelf life and monitoring the microbiological quality of milk to assessing surface contamination of meat, poultry and constitute an approved method in food microbiology for quality control (Torlak *et al.*, 2008). Owing to their small size, Petrifilm plates are convenient for larger sample numbers common in quality control laboratories and require less space in a typical incubator but possess the same limitations as standard plate counting in terms of poor sensitivity and the likelihood of encountering false-negative results from bacteria that may be sublethally injured, precluding accurate enumeration from suspect foods (Feng, 2000). 3M Petrifilm *E. coli*/coliform plates were used to detect and enumerate *E. coli* and coliforms (figure 2). Isolation and characterization of *E. coli*, coliforms, and other Enterobacteriaceae undertaken by selecting five colonies from each *E. coli*/coliform Petrifilm plate based on color, size, and morphology. Colonies were then streaked and purified on nutrient agar and then to Eosin methylene blue and sorbitol MacConkey agar were used for further presumptive confirmation of *E. coli* and coliforms (Ntuli *et al.*, 2016).



**Figure 2** *E. coli* (blue colonies with gas bubbles) and total coliform (red and blue colonies with gas bubbles) on Petrifilm Source: 3M Food Safety, 2017

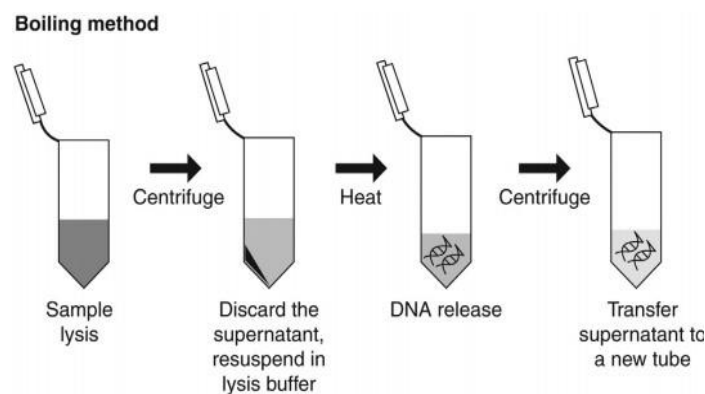
### 2.8.3. Molecular methods of detection

Due to their limitations, conventional methods are now giving way to molecular methods based on DNA analysis, such as polymerase chain reaction (PCR). It has been used to detect foodborne bacterial pathogens such as viable *Escherichia coli* O157:H7 (Lee *et al.*, 2014; Wang *et al.*, 2015). The suitability, time saving and relatively low cost of PCR techniques makes possible to develop sensible and specific assay to detect *E. coli* O157:H7 and other STEC, amenable to the requirements of most health surveillance and food control laboratories (Beneduce *et al.*, 2003).

#### 2.8.3.1. DNA extraction

Good quality DNA is a prerequisite for all experiments of DNA manipulation. To study or manipulate nucleic acids, the DNA must first be extracted from cells. Various techniques are used to extract different types of DNA. Boiling Method in general, DNA extraction methods involve three main steps: cell disruption, DNA extraction, and DNA purification. The boiling procedure is one of the simplest protocols, and largely used for total DNA extraction from microorganisms (Sepp *et al.*, 1994; Reischl *et al.*, 2000; De Medici *et al.*, 2003). This simple method has evolved through the simplification of previous approaches that include the use of detergents as sodium dodecyl sulfate (SDS) or cetyltrimethylammonium bromide (CTAB), enzymes like lysozyme and proteinase K, a phenol-chloroform purification and ethanol or isopropanol precipitation (Wilson *et al.*, 1990; Neumann *et al.*, 1992; Cheng and Jiang, 2006).

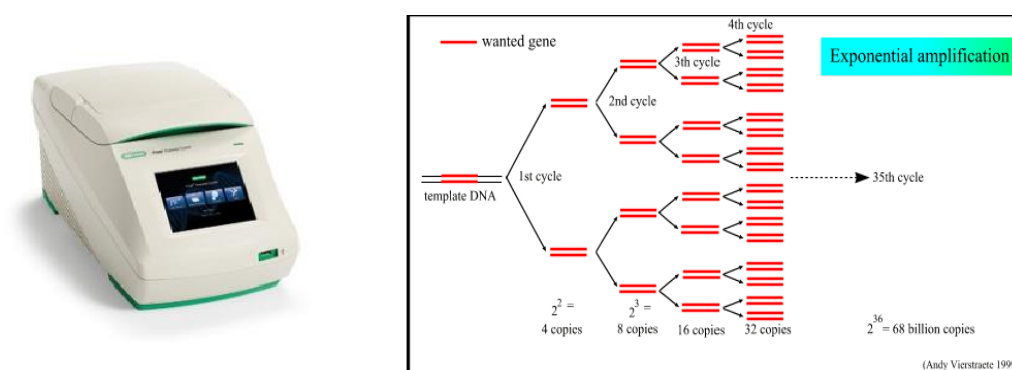
methods is that cells are lysed, cell components, such as lipids and proteins, are denatured in the presence of a detergent (proteins may also be hydrolyzed using a protease), and are removed by organic solvent extraction (figure 3). These protocols have the advantages of having low cost, not requiring specific materials, and of being suitable for extracting DNA from many types of microorganisms. Briefly, portions of individual bacterial colonies are suspended in a lysis buffer containing a detergent, (e.g., 1% Triton X-100 and/or 0.1% Tween 20), and a buffer solution (e.g., 10 mM Tris-HCl, 1 mM EDTA, pH 8). This cell suspension is incubated for 10–15 min at 95–100°C in a heat block or boiling water bath. Then, the tubes are briefly centrifuged to sediment the debris, and the supernatant is ready to be used. Nevertheless, it provides reasonable amounts of DNA that are usually enough to be used in amplification-based methodologies. It is also very easy and inexpensive. The major drawbacks are the low DNA yield and purity that are the result of the detergent usage, and the remaining cell debris that can contaminate. Microbial DNA extraction using the boiling method directly from food samples is also possible, although sometimes it may become challenging. This is due to the possible low numbers of microorganisms present in the sample and also due to PCR inhibitors present in food matrices (e.g., fats, proteins, polysaccharides, and calcium). In other words, the extraction might be affected by the physical, chemical, and biological characteristics of the food material (Wilson, 1997; Giacomazzi *et al.*, 2005).



**Figure 3** Schematic Representation of the Boiling Method Source: Barbosa *et al.*, 2016

### 2.8.3.2. Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a rapid procedure for in vitro enzymatic amplification of specific DNA sequences using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA (Bartlett and Stirling, 2003). Repetitive cycles involving template denaturation, primer annealing and the extension of the annealed primers by DNA polymerase, result in the exponential accumulation of a specific fragment whose termini are defined by 5' end of the primers (figure 4). The primer extension products synthesized in one cycle can serve as a template in the next. Hence the number of target DNA copies approximately doubles at every cycle. (Atawodi *et al.*, 2010)

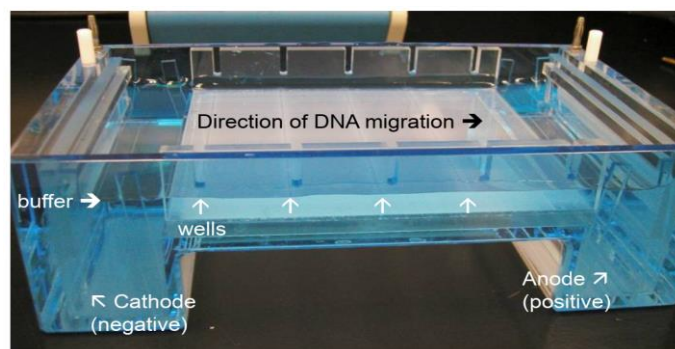


**Figure 4.** Thermal cycler and Polymerase chain reaction conditions Source: (Vanfleteren and Vierstrate, 1999)

Conventional PCR has been used to detect *stx* genes for screening STEC in clinical specimens as well as in food samples including ground beef and raw milk. The technique has proven to be both sensitive and specific. Most PCR assays for detecting STEC employ two pairs of primers (multiplex) specific for *stx1* and *stx2*, respectively. STEC isolate culturing was done by standard methods, including SMAC agar, and *E. coli* was identified by standard biochemical tests screening of *stx1* and *stx2* was performed using primers and amplification conditions as described by (Nielsen and Andersen, 2003). PCR for *eae* was done using the AE13 and AE14 primers and amplification conditions were as described by (Brandal, *et al.*, 2006) Confirmation of *stx1*, *stx2*, and *eae* was done at the National Reference Laboratory for Enteropathogenic Bacteria (NRL) (Brandal, *et al.*, 2012).

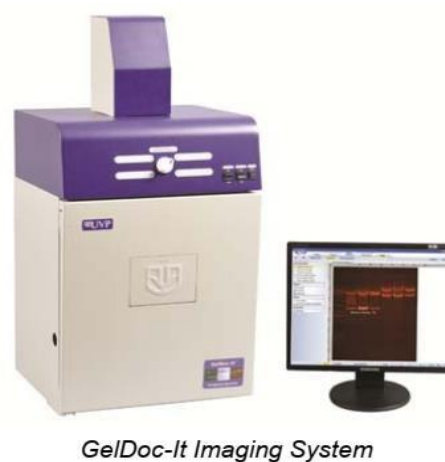
### 2.8.3.3. Agarose gel electrophoresis and Gel imaging

Agarose gel electrophoresis is one of the most common and widely used methods. It is a powerful separation method frequently used to analyse DNA fragments generated by restriction enzymes, and it is a convenient analytical method for determining the size of DNA molecules in the range of 500 to 30,000 base pairs (Surzycki,2000). It can also be used to separate other charged biomolecules such as dyes, RNA and proteins. Since DNA has a strong negative charge at neutral pH, it migrates through the gel towards the positive electrode during electrophoresis (Lee *et al.*,2012) (figure 5).



**Figure 5.** Agarose gel electrophoresis Source: (IMBB, 2013)

Gel imaging and nucleic acid binding dyes are widely used in today's life science laboratories to visualize DNA fragments in agarose gels (figure 6). GelRed™ and GelGreen™ dyes are a new generation of fluorescent nucleic acid gel stains designed to replace the highly toxic Ethidiumbromide EtBr (Gallagher and Wiley, 2010)

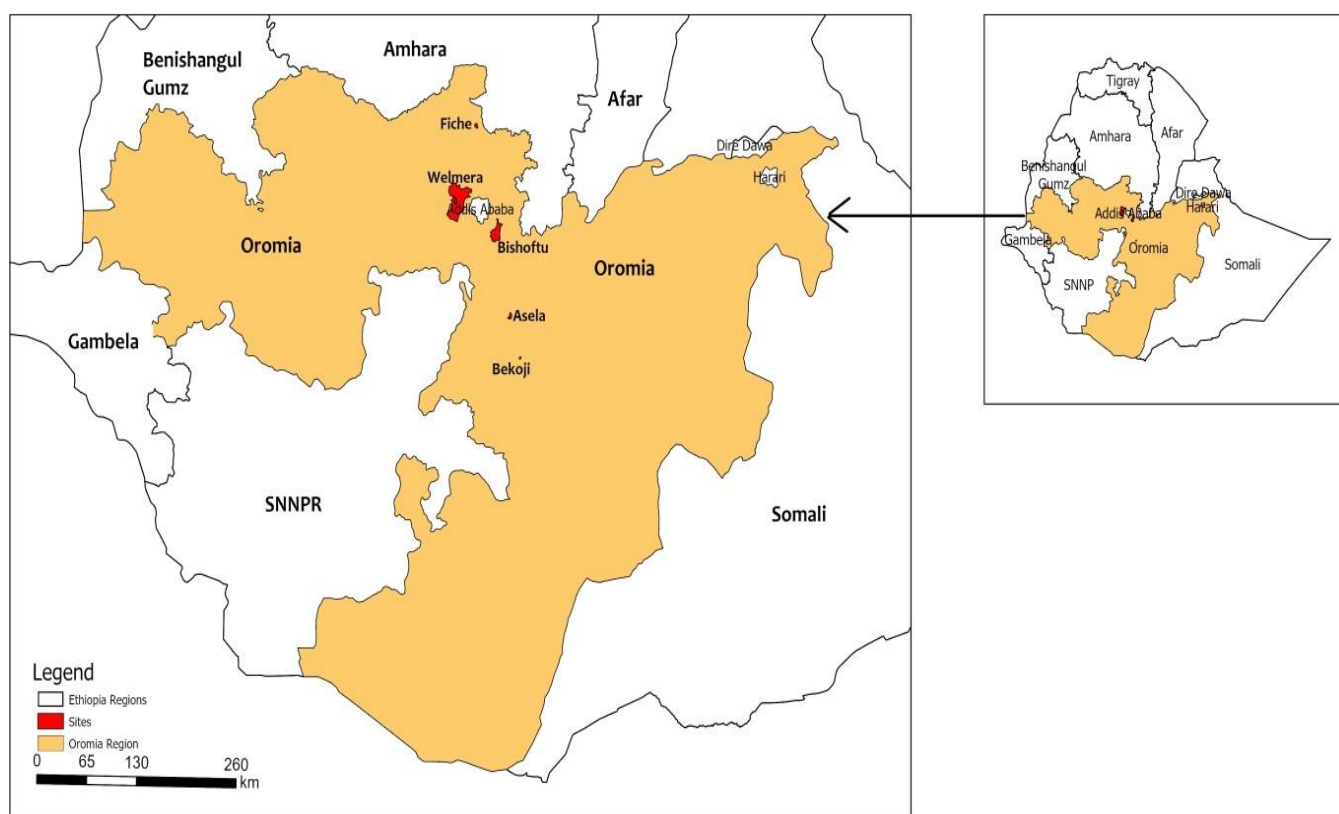


**Figure 6.** GelDoc-It Imaging System Source : (Gallagher and Wiley, 2010)

### 3. Materials and methods

#### 3.1. Study area, design and study period

A cross sectional study was conducted in four selected areas in Oromia regional state namely Wolmera, Deber-zeit, Asela and Selale (Figure 7) from December 2020 to May 2020. The study districts were selected based on milk production potential according to (CSA, 2019). The study areas are located within a radius of 175 km from the capital city, Addis Ababa in an altitude range of 1850 to 4130 meters above sea level. The mean annual rainfall varies from 866 to 1800 mm. The average annual temperature for Wolmera, Deberzeit, Asela and Selale were 6 - 21°C, 18.7 °C, 22.5°C and 21°C respectively.



**Figure 7.** Sampling sites in Oromia maps Source: ENSURE E-Dairy project

#### 3.2. Sample size and sampling technique

##### 3.2.1. Sample size

A 480 representative samples (milk and cottage cheese) were determined based on previously published prevalence of microbial load in milk and milk product. The sample size was calculated using the statistical formula (Daniel, 1999)

$$N = \frac{(Z_{\alpha/2})^2 (1 - p)}{d^2}$$

Where N, is the minimum sample size required

$z=1.96$  at 95% confidence interval

**d**, is margin of sampling error tolerated (5% marginal error was used)

**p**, is an estimate of the prevalence rate for the population, since the overall prevalence of the study area was not known p was taken to be 50% for the calculation, and a total of 384 samples were collected.

$$N = \frac{(1.96)^2 0.5(1-0.5)}{0.0025} = 384$$

In addition, 96 cottage cheeses were also sampled for the microbial assessment and detection of Shiga toxin producing *E. coli*. Purposive sampling was used to select kebeles and stake holders or household based on their production. The total numbers of milks and cottage cheese to be sampled are summarized in the table 1.

**Table 1.** Number of raw milk and pasteurized milk samples collected across the value chains in Oromia region

Sites	Milk value chain				Cottage cheese value chain		
	producer <sup>a</sup>	Collectors <sup>a</sup>	Processor <sup>b</sup>	Retail <sup>b</sup>	Producer	Retail	
Selale	24	24	24	24	12	12	120
Wolmera,	24	24	24	24	12	12	120
Deber-zeit	24	24	24	24	12	12	120
Asela	24	24	24	24	12	12	120
Total milk and cottage cheese samples	96	96	96	96	48	48	480

<sup>a</sup> Raw milk    <sup>b</sup> Pasteurized milk

### 3.2.2. Sampling techniques followed for milk and cottage cheese value chain

#### 3.2.2.1. Sampling of milk and cottage cheese producers

These techniques were designed to give everyone in the target population equal chance of being sampled. When we took the sample which were collected from Oromia region the target

populations (Farmers) are the milk or cottage cheese producers who play a significant role in dairy value chain. The target population were identified using purposively method. In some cases, the purposive design was followed by the random probability method sampling. First the development experts in two identified woredas or kebeles were contacted and potential household farmers listed then the farmers were selected randomly. The criteria for selection of milk producers were willingness of milk producers (farmers) to participate and sign on the consent form, milk production capacity of the farmers, convenience to closed to milk collection center, practiced to process milk to milk products (cottage cheese) and farmer who provide or distribute milk to collection center/factory/milk shop/retailer. Those criteria were used for select farmers in the selected site their availability were beyond the required number. After, getting the list of the farmers, either we got exact or beyond the required numbers. If the numbers of the farmers match with required number of participants, all the farmers were included, but random selection method was employed for those beyond of required farmers (at each sites) to select the final participants. If milk producers produced cottage cheese, the sampling method were addressed the same with raw milk sampling method or the cottage cheese were collected from pre-identified market places

### **3.2.2.2. Sampling technique from milk collection sites or collectors**

In Oromia region, there were more collection sites and most of them drive milk to milk factories which are found in or around Addis Ababa, the capital city. The selection criteria for the milk collection centers were that milk collectors should be collect milk from farmers and distribute as raw primarily to processors, hotels and cafeterias, small milk processors (boiled and soled to consumers) and sell raw of the milk to consumers directly, could be addressed at milk collection centers or supermarkets who took raw milk from farm distributes to consumers. The criteria for milk collector selection were willingness to participate in the research (interviewing, giving raw milk) and sign the consent form, . In case of studied sites where they were limited primary collection centers or points, individual milk collectors from the producer's acts as milk collectors of studied sites, the highest of milk collection capacity of milk collection center should be prioritized, in case of large number of milk collection center. Those milk collection centers or individual fitting the criteria were the target population, but the studied population were identified by several statistical methods. Although, this research was fall in stratified (categories in value chain), systematic random sampling method. Since this research had different categories in terms of value chain (producers, collectors, processors and retailers,

these chains were considered as strata. In some cases where limited milk collectors are available for selection, purposive sampling was applied to cover all milk collectors.

### **3.2.2.3. Sampling technique at milk processors**

Processors are any processing action takers on milk either for the purpose of adding value or for safety purpose. Thus, pasteurized milk collected at the gate of milk pasteurization plants were evaluated under processors or factory. Purposive design and purposive sampling were employed. The criteria to be considered as milk processor were that the processing plant should collect raw milk either from own farm or individual farmers, pasteurize, pack the processed milk and distribute the products to retailers. Management system should be willing to be interviewed, sign the consent form and provide pasteurized milk samples at the gate of the factory. Milk processing plant should be located in selected studied sites Milk processing plant should produced both pasteurized milk and cottage cheese. Milk processors should be recognized by Ethiopian milk and meat institute. Milk processors should have pasteurizer machines and facilities. The list of milk processors in the selected sites was obtained from Ethiopian institute of meat and milk institute. Then, milk processors present in Oromia region were identified together with an expert from Ethiopian meat and milk institute, However, due to limited number of milk processors, all milk processing plants were included in the research.

### **3.2.2.4. Sampling technique at milk retailers**

Milk produced in Oromia region (Selale, Wolmera, Debrezeit and Asella) is directly delivered to milk factories which is found in or around Addis Ababa city. Number of milk shops or supermarkets were considered for the study. Pasteurized milk which was found at country districts was also considered. The selection criteria for retailers were; retailers should sell either pasteurized milk or cottage cheese derived from processing plant or traditionally processed cottage cheese which was derived from farmers to the final consumers. Retailers should be from selected study sites as per the research aim. They were willing to sign the consent form, to be interviewed and to sell their product (both pasteurized milk and cottage cheese) for laboratory analysis. The retailers were registered in the office of income and revenue as tax payer, retailers who sell pasteurized milk and cottage cheese at selling sites and distribute to a number of consumers by their own transportation were preferable. With the help of experts, the retailers were listed from each sub-city or kebele of the towns. To get representative sample, multi-stage random sampling probability method was applied. This means all listed numbers of retailers were clustered to their respective sub-cities (Addis Ababa) and kebeles. Then, from each cluster, required numbers of retailers were selected in random

sampling method. Retailers of cottage cheese sell cottage cheese either at local farm market or local shops. If milk producers produced cottage cheese, the sampling method was addressed the same with raw milk sampling method or the cottage cheese were collected as pre-identified market places

### **3.3. Survey data collection**

Related literatures was reviewed to develop the questionere by identifying the most important risk factors reported previously. The questionnaire was peer-reviewed and refined by ENSURE E-Dairy Researchers and pre-tested in a preliminary survey and was conducted in selected households. The main reason for the preliminary survey is to test the relevance of the questions. This is an effort to ensure that only relevant and well phrased questions are to be posed to the interviewees and also give an opportunity to rephrase some of the questions during the main study.

### **3.4. Survey tool**

The survey tool was an e-questionnaire data collecting platform called Kobo Toolbox (<https://www.kobotoolbox.org/>) and all questions have been already uploaded into the tool. Android phone was used for administrating the questions in field off-line and upon return the respondent responses were uploaded online when a Wi-Fi internet was accessed. See the developed survey questionnaires from annexes. The questionnaire was initially prepared in English language and later translated into local language and then loaded to Kobotoolbox (field data collection tool) then a face-to-face interview was conducted using semi-structured questionnaire to collect information on the knowledge and practices of the milk handling, storage and hygiene (Annex 2-7).

### **3.5. Sample handling and transportation**

Sample handling and transportation was implemented in accordance with milk and milk products sampling guidance of ISO 707:2008 as adapted by Ethiopian Standard Agency (ESA). Raw milk was collected from producers and collectors whereas pasteurized milk was collected from processors and retailers. Cottage cheese was from producers (farmers) and retail market. Briefly, sterile cotton soaked in 70 % ethanol was used for hand disinfection immediately prior to sample collection to reduce cross contamination. Samples of raw milk (200 ml) and cottage cheese (250 g) were collected in sterile screw-capped clean plastic bottles (250 mL capacities) and zipper polyethylene plastic bag, which were then securely capped secured, labeled with

permanent markers, and transported in thermoelectric portable refrigerator freezer to Addis Ababa University-Center for Food Science and nutrition laboratory within 24/48 hrs of sampling. Samples were analyzed immediately or kept in freezer at -18°C until analysis.

### 3.6. Assessment of microbiological safety

#### 3.6.1. Total bacterial count (TBC)

The TBC of milk and cottage cheese samples was determined as per the procedures given in the (FDA, 2001) using pour plate method using plate count agar (Oxoid, UK). One ml of milk sample was serially diluted in 9 ml of Butterfield's phosphate-buffered dilution water (ratio of 1:10) up to five and seven dilutions for pasteurized and raw milk, respectively. Sterile duplicate glass petri dishes of 90 mm were labelled according to the dilution index. One ml of each dilution was aseptically withdrawn using a sterile measuring pipette and added into the center of 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<sup>-5</sup> dilution. This was followed by pouring about 15-20 ml of standard plate count agar. The aliquot and the medium 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 yielding between 25 and 250 colonies were counted. Colony counts were made using colony counter and the number of bacteria in millilitre milk was calculated by the following formula given by (FDA, 2001).

$$N = \frac{\sum c}{V \times 1.1 \times d}$$

Where:

$\sum C$  is the sum of the colonies counted on the two dishes retained from two successive dilutions, at least one of which contains a minimum of 10 colonies

V is the volume of inoculum placed in each dish, in milliliters;

d is the dilution corresponding to the first dilution retained [d = 1 when the undiluted liquid product (test sample) is retained (ES ISO 7218, 2015)]

### 3.6.2. Coliform and *E. coli* count

The total coliforms and *E. coli* count of milk samples were done as per the procedures given in the (Food Safety, 2015). Thus, 1 millilitre of milk sample was serially diluted in 9 ml of saline up to five and third dilutions for raw and pasteurized milk, respectively. Then the prepared dilution was added onto an *E. coli* coliform count plate Petri film after vortex the sample to ensure that the sample was homogenized for quantification of coliforms (Food Safety, 2015). Cottage cheese samples were suspended in saline in sample bags; 10 gram of the sample was taken and mixed with 90 ml of saline by hand until homogenized. This homogeneity represents the  $10^{-1}$  dilution. Then, the top film was lifted, and 1 ml of sample suspension was dispensed onto the inoculation area. The Petri film plates were incubated in a horizontal position at  $35 \text{ }^{\circ}\text{C} \pm 1 \text{ }^{\circ}\text{C}$  for 24 hours  $\pm 2$  hours. The plates were incubated for an additional 24 hours  $\pm 2$  hours (48 hours  $\pm 4$  hours total) until colonies of sufficient size to count are observed. The total coliform count consisted of both red, and blue colonies associated with gas and blue colonies with a gas bubble was counted as *E. coli* (3M Food Safety, 2017).

$$N = \sum C \times V \times d$$

Where:

$\sum C$  is the sum of the colonies counted on the two petrifilms

V is the volume of culture Plates

d is the dilution corresponding to the first dilution retained [d = 1 when the undiluted liquid product (test sample) is retained (3M Food Safety, 2017).

### 3.6.3. Isolation and identification of STEC and *E coli* O157:H7

The isolated *E. coli* (blue colonies with a gas bubble) were sub cultured on Eosin Methylene Blue agar (EMB) (Oxoid, UK) using streak plating technique and then incubated at  $37^{\circ}\text{C}$  for 24 h the colonies with metallic sheen on EMB agar which is typical for *E. coli*. The red colonies with gas were either coliform or suspected as *E. coli* O157:H7. Since most *E. coli* O157:H7 strains are atypical, they are glucuronidase negative; they did not produce a blue color, so Red colonies with gas were transferred to sorbitol MacConkey agar supplemented with cefixime and potassium tellurite (CT-SMAC) and incubated at  $37^{\circ}\text{C}$  for 24 h to check the presence of colorless colonies (inability to ferment sorbitol). Then the typical colonies on EMB and CT

SMAC were transferred to nutrient broth and incubate at 37 °C overnight for further confirmation by PCR (Figure 8)

### **3.6.4. Confirmation of Shiga toxin *E. coli***

#### **3.6.4.1. DNA extraction**

The culture was grown in nutrient broth at 37 °C overnight. Exactly 1.5 ml of the culture was spun by centrifugation at 5000 rpm for 10 min. The bacterial pellet was lysed by adding 50 µl of double distilled water and boiled in a water bath at 95 °C for 10 minutes. The lysate was then centrifuged again as before and 50 µl of the supernatant used directly as template for PCR (De Medici *et al.*, 2003).

#### **3.6.4.2. Detection of virulent genes**

After extraction of bacterial DNA, *E. coli* isolates were subject to PCR for the presence of virulence genes *stx1*, *stx2* and *eae*. According to the annealing temperatures of the different primers described below (Table 2) the PCR experiments were carried out by optimizing the protocols. In order to detect the presence of *stx1* genes of STEC strains, a reaction was set up in a 25µl mixture containing nuclease free water (8 µl), both forward and reverse primers (2 µl), Gotaq master mix (Promega, USA ) (12µl), and template (3µl). The reaction mixture was amplified with an initial denaturation of 1 cycle for 3 min. at 95°C; 35 cycles each consisting, 40 s at 95°C, 40 s at 55°C, 30 s at 72°C; and a final extension of 1 cycle for 8 min. at 72°C.

In order to detect the *eae* (intimin) gene of STEC strains, a reaction was set up in a 25µl mixture containing nuclease free water (8 µl), both forward and reverse primers (2 µl), Gotaq master mix (12µl), and template (3µl). The reaction mixture was amplified with an initial denaturation of 1 cycle for 3 min. at 95°C; 35 cycles, each consisting of 40 s at 95°C, 60 s at 55°C and 60 s at 72°C; and a final extension of 1 cycle for 10 min. at 72°C.

*Stx2* gene of STEC strain detection reactions were set up in a 25µl reaction tube in a PCR master mix containing nuclease free water (8 µl), both forward and reverse primers (2 µl), Gotaqmaster mix (12µl), and template (3µl). The reaction mixture was amplified with an initial denaturation of 1 cycle for 3 min. at 95°C; 30 cycles, each consisting of 60 s at 95°C, 60 s at 55°C and 60 s at 72°C; and a final extension of 1 cycle for 8 min. at 72°C using by a thermocycler (BIO-RAD T100™ Thermal cycler 621BR43010, Singapore).

**Table 2:** Primer gene sequence and PCR conditions

Target gene	Primer	Nucleotide sequence 5' to 3'	Pathogenic <i>E. coli</i> strain	Product size (Bp)	Reference
<i>Eae</i>	EAE1	F:AAACAGGTGAAACTGTTGCC	EPEC/ EHEC	490	(Khan <i>et al.</i> ,2002)
	EAE2	R:CTCTGCAGATTAACCTCTGC			
<i>Stx1</i>	EVS1	F:ATCAGTCGTCACACTCACTGGT	STEC/EHEC	110	(Pal <i>et al.</i> , 1999)
	EVC2	R:CTGCTGTCACAGTGACAAA			
<i>Stx2</i>	EVT1	F:CAACACTGGATGATCTCAG	STEC/EHEC	350	(Khan <i>et al.</i> , 2002)
	EVT2	R:CCCCCTCAACTGCTAATA			

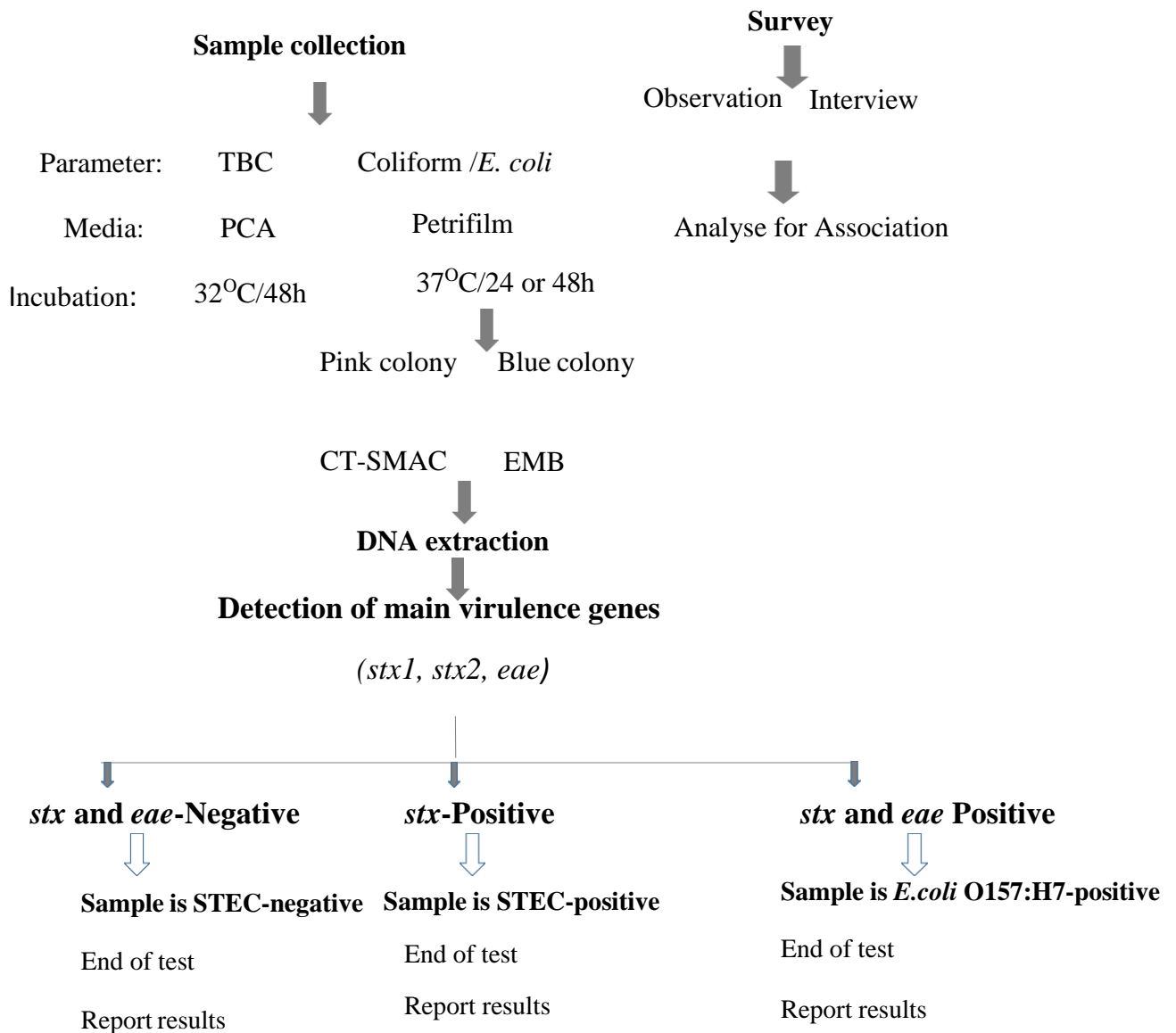
### 3.6.4.3. Agarose gel electrophoresis

Amplified PCR products (5-7µl) were analyzed using agarose gel electrophoresis. The 1.5% agarose gel in 1X TAE (Tris-acetic acid EDTA) running buffer was stained with 10µl of gel red (B7025S, New England) for 40 minutes at 120V. A 100bp molecular weight ladder marker (product no. N32315), positive and negative controls were also employed. The products were visualized with ultraviolet illumination and imaged with gel documentation system (*El-baz et al.*, 2017). Band views of 490,110 and 350 base pairs (bps) were used to determine the virulent genes of STEC and *EC* O157:H7 isolates as *intimin (eae)*, *shiga toxin1 (stx 1)* and *shiga toxin2 (stx 2)* respectively (Table 2).

### 3.6.4.4. Antimicrobial susceptibility testing

The antimicrobial susceptibility/resistance profiles of the bacterial isolates were determined using Kirby – Bauer – disk diffusion method (Quinn *et al.*, 2002). Disks impregnated with the following antibiotics were used: Trimethoprim (5µg), chloramphenicol (30µg), ciprofloxacin (5 µg), ampicillin (10 µg), neomycin (10 µg), tetracycline (30 µg), oxytetracycline (30 µg) compound sulfonamides (30 µg), clindamycin (10 µg), Norfloxacin (10 µg) and streptomycin (25 µg). Pure bacterial colonies grown in Tryptic soya agar were inoculated into 7 ml of Tryptophan soya broth and incubated at 37 °C for 8 hours until turbidity was seen which was compared to the 0.5 McFarland standards Mueller – Hinton Agar was used as plating medium. A McFarland Standard is a chemical solution of barium chloride and sulfuric acid; the reaction between these two chemicals results in the production of a fine precipitate, barium sulfate. When shaken well, the turbidity of a McFarland Standard is visually comparable to a bacterial

suspension of known concentration (McFarlan,1907). Fifteen minutes after inoculation of the plates, the antibiotic impregnated disks were applied on the surface of inoculated plates with sterile forceps. All the disks were gently pressed down onto the agar with forceps. The plates were inverted and then incubated aerobically for 18 hours at 37 °C. The diameters of the zones of inhibition were measured to the nearest whole millimetre using the transparent ruler and were interpreted as susceptible, intermediate and resistant (CLSI, 2008).



**Figure 8.** Detection of shiga toxin producing *Escherichia coli*

### **3.7. Data analysis and interpretation**

The data obtained were classified, filtered and coded using Microsoft Excel® 2010. The data were then exported to SPSS version 20.0 (SPSS IBM) software package for appropriate statistical analysis. Logistic regression was used to analyze microbiological data. Odds ratio (OR) was used to indicate the degree of association among different risk factors. In all analyses, p values less than 0.05 were considered as statistically significant.

### **3.8. Data quality control**

Laboratory data quality was maintained by using instruments, reagents, and chemicals from reputable companies. Procedures for sample collection, microbiological analysis and PCR confirmation were conducted using validated protocols and standard operational procedures developed by the ENSUREproject. Interpretation and evaluation of test results and quality controls were applied as recommended by the manufacturers. Reference strains of STEC (NCCB100282, Netherlands) were periodically used for quality control during the microbiological analysis. Moreover, DNA extract from standard Shiga toxin *E. coli* and nuclease free water were used as positive and negative controls in each PCR run.

### **3.9. Ethical approval and informed consent**

Ethical approval of the research was obtained from the College of Natural and Computational Sciences Institutional Research Ethics Review Board (CNS-IRB), Addis Ababa University (AAU) with reference no. SUA/ADM/R 1/29. Survey data was collected after explaining the purpose of the study to each study participant and obtaining verbal informed consent. The original information sheet developed in English language was translated into the two local languages (Amharic and Afaan oromo) of the respondent. (Annex 1 for consent form).

## 4. Results and Discussion

### 4.1. Microbiological quality of raw milk, pasteurized milk, and cottage cheese

#### 4.1.1. Total bacteria counts (TBC) in raw and pasteurized milk

The total bacterial counts (TBC) of raw and pasteurized milk samples ranged from 5.61 log cfu/ml to 9.73 log cfu/ml and 4.21 log cfu/ml to 8.61 log cfu/ml, respectively. The mean TBC ( $7.17 \pm 0.06$  log cfu/ml) bacterial counts of raw milk was considerably higher as compared to the mean of pasteurized milk samples ( $5.93 \pm 0.05$  log cfu/ml) and the raw milk showed statistically significant difference from pasteurized milk at ( $P < 0.05$ ) (Table 3).

**Table 3.** Mean of TBC, TCC and *E. coli* (log<sub>10</sub> cfu/ml) in raw and pasteurized milk

Bacteria	Milk products	
	Raw milk	Pasteurized milk
TBC	$7.17 \pm 0.06^b$	$5.93 \pm 0.05^a$
TCC	$5.53 \pm 0.11^b$	$3.36 \pm 0.16^a$
<i>E. coli</i>	$1.56 \pm 0.12^b$	$0.97 \pm 0.11^a$

Total bacterial count (TBC), Total coliform count (TCC)

Means followed by different superscript letters within a column are significantly different from each other at ( $P < 0.05$ ).

In this study, the mean TBC result of raw milk was less than the report of (Asaminew, 2010) which was 7.58 log cfu/ml in Bahir Dar Zuria and Mecha districts, Ethiopia. Another report by (Worku *et al.*, 2012) showed total bacterial counts of 7.36 -7.88 log cfu/ml of raw milk in Borana, Ethiopia. The value of total aerobic bacterial count for present study revealed lower than that reported by (Tola, 2002) in Eastern Wollega that reported average count of  $7.4 \times 10^6$  cfu/ml of milk. (Tassew and Seifu, 2011) reported an overall mean of 7.58 log cfu/ml in BahirDar. The findings of the current study corroborate with those obtained by (Solomon *et al.*, 2013) similarly reported TBC of 7.07 log cfu/ml in milk samples obtained from Debre Zeit town; while, this study had higher counts than (Godefay and Molla, 2000) reported 6.0 log cfu/ml of TBC in milk samples collected from selected dairy farms in Addis Ababa. In Tanzania ( Ngasala *et al.*, 2015; Karimuribo *et al.*,2015) showed a bacterial count of  $5.4 \times 10^6$  and  $3.3 \times 10^5$  cfu/ml TBC values which is lower compared to the current study; in addition, a study in Rwanda showed TBC mean values of  $1.5 \times 10^6$  CFU/ml in farmer's milk. Similar results were found by (Doyle *et al.*,2015); they also recorded TBC mean values of  $1.5 \times 10^6$  CFU/ml.

The mean TBC of the pasteurized milk samples in this study was lower than the report of (ICMSF,1998), which reported a TBC in pasteurized milk of  $7.5 \times 10^7$  to  $1.24 \times 10^8$  cfu/ml and the result of (Tamirat, 2018) who found 2.42 log cfu/ml. Pasteurization has the advantage of reducing the transmission of disease causing bacteria and improves the keeping quality of milk. Pasteurized milk presents little health hazards. Nevertheless, several food-borne disease outbreaks have been linked to pasteurized milk and traced to inadequate pasteurization, post pasteurization contamination or abuse of storage temperature (ICMSF, 1998). The TBC (total bacterial count) of the pasteurized milk samples from different locations in Bangladesh was reported to range from  $1.1 \times 10^2$  to  $1.8 \times 10^3$  cfu/ml, slightly lower than that recommended by the Bangladesh Standards and Testing Institution (not exceeding 20,000 cfu/ml) According to (Sourav *et al.*, 2014) 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.

#### **4.1.2. Total coliforms counts (TCC) in raw and pasteurized milk**

In this study, the mean total coliform count (TCC) of the raw and pasteurized milk was  $5.53 \pm 0.11$  log cfu/ml and 3.36 log cfu/ml respectively (Table 3). Total coliform count (TCC) of the raw milk in this study was higher than that obtained by (Tamirat, 2018) who found TCC of 3.59 log cfu/ml and the result reported (mean coliform count of 1.82 log cfu/ml) by (Solomon, 2013) in Debre zeit, Ethiopia. The result of the current study is also much greater than a report by (Mesfine *et al.*, 2015) a TCC of 1.24 log cfu/ml. Furthermore, these results were higher than that reported those done in raw cows' milk by (Hundie, 2015) where coliform counts in milk from Aneno, Gulgula and Dongora districts of southern region of Djibouti was 3.8 log cfu/ml and and by (Kas *et al.*, 2013) from the central part of Ivory coast (2.7 log cfu/ml). However, the current result is lower than 8.58 log cfu/ml of TCC reported in the central high lands of Ethiopia (Gemechu, 2016). Another study showed mean coliform counts of 6.63 log cfu/ml of raw milk in Hawassa (Korma *et al.*, 2018). Moreover, the mean level of total coliform counts in our study was lower than the findings reported by (Tankoano *et al.*, 2016) at Ouagadougou (Burkina Faso) (8.95 log cfu/ml). The presence of high numbers of coliforms in milk, which is mainly associated with unclean udder and teats arise from a variety of sources such as manure, soil, food, personnel and even water and thus associated with unclean udder and teats, provides an index of hygienic standard used in the production of milk. Increased level of coliforms suggests that the milk and cottage cheese was processed in unhygienic conditions, which

increase risk for contamination with foodborne pathogens.

In this study, the mean TCC in the pasteurized milk was 3.36 log cfu/ml which was comparatively lower than 6.20 log cfu/ml, 6.05 log cfu/ml and 5.38 log cfu/ml) in studies conducted by (Korma *et al.*, 2018) in Hawassa and (Asaminew and Eyasu, 2011) in Bahr Dar, However, mean TCC of  $3.1 \times 10^5$  cfu/ml reported by (Aberra, 2010) is lower than the result of the recent study. The possible reasons for higher TCC might be the mode of transport to reach the market and managed by people with mostly unsatisfactory knowledge on the keeping quality of milk and the sources of water for sanitation were poor quality. All these conditions favour the introduction and multiplication of bacteria in the milk. These bacteria will reach the consumer through consumption of improperly boiled milk and improperly treated/prepared milk or cheese since milk is also consumed through both of these forms. Coliform bacteria counts of pasteurized milk showed high numbers than those reported by (Wanjala *et al.*, 2017) 0.10 log cfu/ml in Nairobi and  $2.8 \times 10^4$  cfu/mL in South Africa (El-Diasty and El-Kaseh., 2008). The lower coliform counts might be due to hygienic quality of raw milk, proper pasteurization process, good packaging and good storage conditions. This agreed with (SSMO, 2005) who reported that the coliform counts standards for pasteurized milk should be not exceeding 10 cfu/ml.

The mean of TCC for the retailers' pasteurized milk ( $4.31 \pm 0.24$  log cfu/ml) was higher than processors ( $2.42 \pm 0.18$  log cfu/ml) this may be due to poor quality. Producers ( $5.41 \pm 0.16$  log cfu/ml) and collectors ( $5.66 \pm 0.15$  log cfu/ml) raw milk had no significant difference in the TCC at ( $p < 0.05$ ) (Table 4). The TCC of milk samples obtained from producers in the present study was less than the result reported by (Tadesse and Bacha, 2014), which showed the overall mean counts of total coliforms of  $5.85 \pm 0.483$  and  $5.91 \pm 0.19$  log cfu/ml for smallholder and dairy farms, respectively.

The overall mean of coliform count from 30 milk samples collected from Shambu, Fincha and Kombolcha was  $5.6 \pm 0.38$ ,  $5.7 \pm 0.09$  and  $5.4 \pm 0.33$  log cfu/ml, respectively (Demissu, 2014) which was higher than the results of this study, but (Tsfay *et al.*, 2013) reported an average coliform and *Escherichia coli* counts from dairy farms  $4.13 \pm 0.76$  and  $6.2 \pm 0.418$  log cfu/ml, respectively had lower than the current study. The mean coliform count of milk samples collected at 39 samples taken from farmers and 45 samples taken from dairy cooperatives in Bahir Dar Zuria and Mecha district was  $4.41 \pm 0.16$  and  $4.55 \pm 0.15$  log cfu/ml, respectively which was also lower than the current study. On the other hand, the total mean coliform counts of milk collected from three producers was 6.57 logcfu ml<sup>-1</sup> (Zelalem and Bernard, 2006) which was also higher than the current study. According to (Dehinenet *et al.*, 2013), 60 milk samples from 6 districts collected from Amhara and Oromia showed a mean total coliform count of  $3.0 \times 10^4$  cfu/ml; the study conducted in Debre Brhan, Selale and Holeta showed an average coliform count of 3.2 log cfu/ml in whole milk whole milk samples (Ghilu *et al.*, 2012) this was lower than the current study.

#### **4.1.3. *Escherichia coli* (*E. coli*) counts in raw and pasteurized milk**

The presence of *E. coli* in raw milk, pasteurized milk and cottage cheese were (36.2%) which was detected in 174 samples out of 480 samples of milk and cottage cheese samples. The presence of *Escherichia coli* in milk and cottage cheese is a common indicator of faecal contamination it was isolated from 102 out of 192 raw milk samples (53.1%). In this study, raw milk samples showed significantly higher ( $1.56 \pm 0.12$  log cfu/ml) *E. coli* counts than pasteurized milk ( $0.97 \pm 0.11$  log cfu/ml) (Table 3). Samples did not comply with the Ethiopian Standard which recommended the *E. coli* to be nil in marketable dairy products. The sources of *E. coli* in the raw cow milk may be from contaminated udders, contaminated sources of water, poor sanitation practices, contaminated containers, and poor hygiene of milk handler.

This current finding was comparable with the finding of (Iqbal *et al.*, 2004) 40.7% and (Megersa, *et al.*, 2019) 42%. However, the current finding was much higher than the finding of (Demme and Abegaz, 2015) which was 18.6% of *E. coli* detected from raw milk. Another report revealed that 10% of raw milk samples under study were contaminated with *E. coli* (Tasci, 2011) in Khartoum State and (Crump *et al.*, 2002) reported that 13% of samples were contaminated with *E. coli*.

In the current study from 192 pasteurized milk samples 69 samples (35.9%) were contaminated by *E. coli*. This study is higher *E. coli* contamination on pasteurized milk rather than other

studies, from 100 pasteurized milk samples 9 (9%) were positive for *E. coli* (Vahed, 2011) in Sari city, Iran. Another study confirmed that *E. coli* were not found in pasteurized milk (Bedasa *et al.*, 2018).

*E. coli* counts showed significant difference along the value chain at ( $p < 0.05$ ). (Table 4) The raw milk samples collected from producers and Pasteurized milk collected from retailers had means ( $1.17 \pm 0.15$  log cfu/ml and  $1.33 \pm 0.18$  log cfu/ml) higher next to raw milk collectors with ( $1.96 \pm 0.17$  log cfu/ml). Pasteurized milk samples collected from processors had the lowest *E. coli* count ( $0.60 \pm 0.1$  log cfu/ml). TBC, the results of TCC and *E. coli* of cottage cheese samples did not show statistically significant difference between the producer and retailer.

**Table 4.** Mean of TBC, TCC and *E. coli* (log<sub>10</sub> cfu/ml or g) in milk across the value Chain  
Value chain Actors

Bacteria	Raw milk		Pasteurized milk	
	Producer	Collector	Processor	Retailer
TBC	$7.10 \pm 0.09^c$	$7.24 \pm 0.07^c$	$5.71 \pm 0.06^a$	$6.16 \pm 0.08^b$
TCC	$5.41 \pm 0.16^c$	$5.66 \pm 0.15^c$	$2.42 \pm 0.18^a$	$4.31 \pm 0.24^b$
<i>E. coli</i>	$1.17 \pm 0.15^b$	$1.96 \pm 0.17^c$	$0.60 \pm 0.10^a$	$1.33 \pm 0.18^b$

Total bacterial count (TBC), Total coliform count (TCC)

Means followed by different superscript letters within a row are significantly different from each other at ( $P < 0.05$ ).

There was significant difference among TBC along the milk value chain at  $P < 0.05$ . The study indicated that there is high TBC count of raw milk samples at producers and collectors level with mean counts of  $7.1 \pm 0.09$  log cfu/ml and  $7.24 \pm 0.07$  log cfu/ml, respectively (Table 4).

Pasteurized milk samples from retail shops had lower mean ( $6.16 \pm 0.08$  log cfu/ml) and pasteurized milk samples collected from processors had the lowest TBC ( $5.71 \pm 0.06$  log cfu/ml) as compared to that of other value chain actors. There was significant difference between the TBC of pasteurized milk samples collected from processors and retailers at  $P < 0.05$ , but there was no significant difference between the TBC of raw milk samples collected from the producers and collectors

In other studies, also producer's milk showed higher TBC, For instance, from a total of 100 raw

cow milk samples, 88 were from individual farmers and 12 were collected from dairy farms in Jimma, the overall mean TBC was  $8.7 \pm 1.34$  and  $8.27 \pm 0.98$  log cfu/ml (Teshome and Ketema, 2014). The mean TBC of 39 milk samples collected from producers and 45 samples from dairy cooperatives in Bahir Dar Zuria and Mecha district, North-western Ethiopia, was  $7.61 \pm 0.12$  and  $7.56 \pm 0.13$  log cfu/ml, respectively, which was higher than that of the current study. (Tesfay *et al.*, 2013) reported an average TBC of  $5.84 \pm 0.629$  log cfu/ml in dairy farms in Dire Dawa town, Eastern Ethiopia. This finding is lower than done in Tanzania by previous workers (Rwehumbiza *et al.*, 2013; Fortunate, 2013; Kweka, 2002; Kivaria *et al.*, 2006) and in Ghana (Addo *et al.*, 2011) and higher than the study done in Sudan (Adil and Iman, 2011).

In the present study on total bacterial count (TBC) in raw and pasteurized milk as compared to Ethiopian standard 92.7% of raw milk had higher counts than that recommended amount (not exceeding 1,000,000 log cfu/ml) (ESA, 2009) and Out of 192 raw milk samples, only 7.3 % of the raw milk samples had less than 1,000,000 log cfu/ml of TBC. Out of 192 pasteurized milk samples 83.4% had higher than that recommended by Ethiopian standard (not exceeding 100,000 log cfu/ml) (ESA, 2009), and East Africa standard (not exceeding 30,000 log cfu/ml) (EAS, 2009). But only 16.6% of the pasteurized milk samples had less than 100,000 log cfu/ml of TBC (Table 5).

**Table 5.** Total Baterial count (TBC) in raw and pasteurized milk as compared to ESA standards Ethiopian standard (ESA 3460:2009)

Number of samples	Quality	Raw milk				Pasteurized milk		
		Very good	Good	Bad	Very bad	Very good	Good	Shall not be offered for sale
	Counts per ml	0 –200,000	200,000 – 1,000,000	1,000,000 – 2,000,000	2,000,000 and over	< 50,000	50,000- 100,000	>100,000
192	Total bacterial count	None	14(7.3)	37(19.3)	1 41(73.4)	16(8.3)	16(8.3)	160(83.4)

In the present study the TCC in the raw milk samples ranged from < 10 log cfu/ml to 10.17 log cfu/ml. About 78.1% of raw milk samples was higher than that recommended by Ethiopian standard (not exceeding 50,000)(ES, 2009) and East AfricanStandards (not exceeding 50,000) (EAS, 2006). However, 21.9% of the raw milk samples had TCC of <50,000 log cfu/ml. In pasteurized milk the count ranged from < 10 log cfu/ml to 9.33 log cfu/ml; 79.2% of pasteurized milk samples showed higher values than that recommended by Ethiopian standard (ES, 2009) (not exceeding 10 cfu/ml) and East African Standards (EAS, 2006). Only 20.8% of the pasteurized milk samples had <10 log cfu/ml (Table 6). In EAC, the recommended levels are  $5.0 \times 10^4$  Cfu/ml for TCC. Analyses performed in Tanzanian the levels of coliform count were  $1.4 \times 10^6$  cfu/ml and  $4.2 \times 10^6$  cfu/ml (Ngasala *et al.*, 2015; Karimuribo *et al.*, 2015).

**Table 6.** Total coliform count (TCC) in raw and pasteurized milk as compared to ESA standards

Number of samples	Quality Counts per ml	Raw milk			Pasteurized milk		
		Very good 0 – 1,000	Good 1,000 – 50,000	Bad 50,000 – 500,000	Very bad 500,000 and over	Pass Not more than 10 per ml	Fail Above 10
192	Total coliform count	5(2.6)	37(19.3)	66(34.4)	84(43.7)	40(20.8)	152(79.2)

Ethiopian standard (ESA 3460:2009)

#### 4.1.4. Total bacteria counts in cottage cheese

The result of TBC of cottage cheese is presented in (Table 7). There was no statistically significant difference between producer and retailer cottage cheese on total bacterial count.

**Table 7.** Mean of TBC, TCC and *E. coli* (log<sub>10</sub> cfu/g) in producer and retailer cottage cheese

Bacteria	Cottage cheese	
	Producer	Retail
TBC	6.11 ± 0.08	6.1 ± 0.04
TCC	1.85 ± 0.37	1.6 ± 0.37
<i>E. coli</i>	0.38 ± 0.22	ND

The mean  $6.11 \pm 0.08$  and  $6.1 \pm 0.04$  collected from producer and retailer, respectively, was lower than 8 log cfu/g that was reported by (Ashenafi, 2006) and (Zelalem *et al.*, 2007) that revealed 7.9 log cfu/g and 8.8 log cfu/g, respectively. Whereas, the mean 5.38 log cfu/g reported by (Ashenafi *et al.*, 2016) was lower than the current study. In this study the range of TBC counts for cottage cheese was from 7.42 log cfu/g to 9.64 log cfu/g and 5.42 to 7.22 log cfu/gm for producer and retailer, respectively.

#### **4.1.5. Total coliforms counts in cottage cheese**

The counts of total coliforms in cottage cheese ranged from  $< 10$  log cfu/gm to 11.96 log cfu/gm. Out of 96 cottage cheese samples 37.5 % was  $>10$  which is above the recommended by East African Standards (not exceeding 10 cfu/ml) (EAS, 2019) (Table 6). The mean TCC of cottage cheese in the current study was  $1.85 \pm 0.37$  and  $1.6 \pm 0.37$  log cfu /gram for producer and retailer cottage cheese respectively (Figure 4). Earlier works carried out in different parts of the country showed high results than the present study (Yilma *et al.*, 2005)

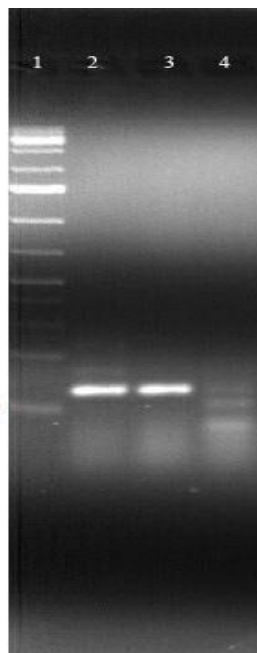
reported the mean 5.68 log cfu/gm which was much higher than the present result. The report of (Birhanu *et al.*, 2013) also showed high mean count of 5.709 log cfu/gm of TCC in cottage cheese and also (Zelalem *et al.*, 2007) and (Ashenafi *et al.*, 2016) show high total coliform in cottage cheese by the mean of 4.4, log cfu/gm and 5.58 log cfu/gm respectively.

#### **4.1.6. Level of *Escherichia coli* (*E. coli*) in cottage cheese**

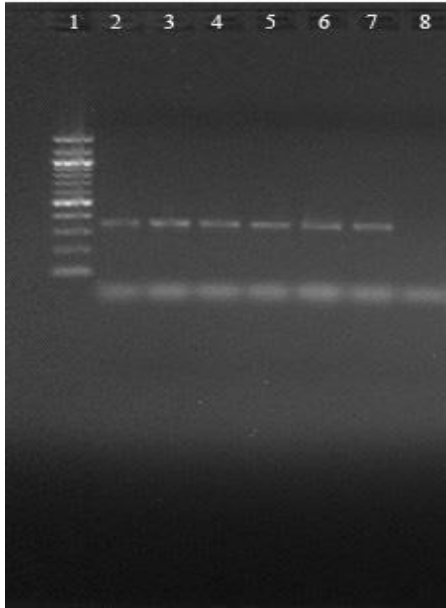
In this study from 96 cottage cheese samples 3 of them (3.1%) were positive for *E. coli*. A study conducted in south western Nigeria (Ogunshe *et al.*, 2008) showed (24.3%) *E. coli* in cottage cheese. In another study, out of 200 bacteriologically examined samples the highest isolation was from cheese (40%) (Bedasa *et al.*, 2018). In Ethiopian cottage cheese, complete inactivation of the organism occurred after 20 and 40 min of cooking at 70 °C, indicating that if there is under treatment of heat, the cheese can act as source of *Escherichia coli* (Yilma *et al.*, 2015).

## 4.2. Prevalence of STEC and EHEC strains in milk and cheese

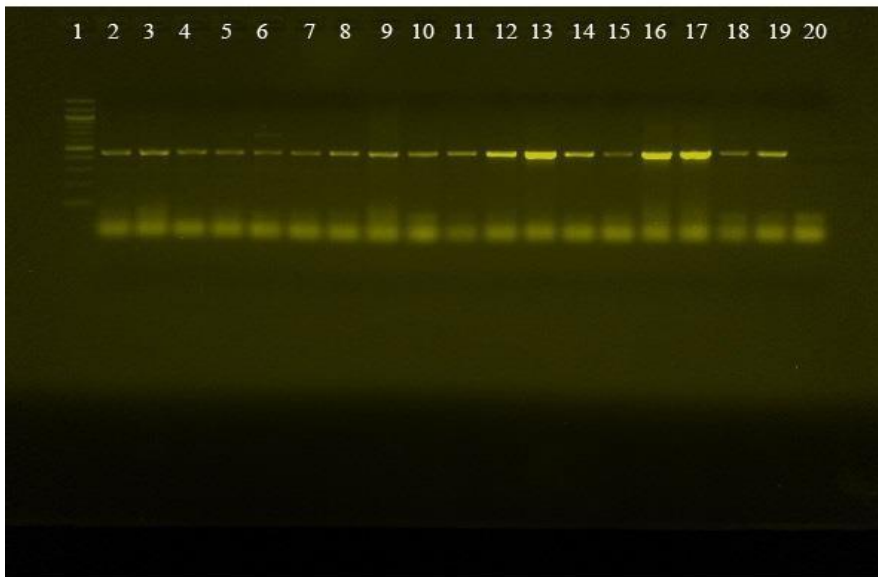
In the present study out of 480 samples of raw milk, pasteurized milk and cottage cheese that cultured separately on selective media a total of 142 (29.8%) and 81(16.8%) isolates were suspected as STEC and *E. coli* O157:H7 on EMB and SMAC Agar, respectively. The suspected colonies of STEC were detected in all the three types of samples (i.e. raw milk, pasteurized milk and cottage cheese). From the suspected isolates 58 (12%) were harboured *stx1*, *stx2*, and *eae* genes confirmed in (20.31%) raw milk, 17(8.85%) pasteurized milk and 2(2.08%) cottage cheese respectively (Table 8). The virulence genes of *stx1* were detected in 1(0.7%) (Figure 9) and *stx2* in 39 (27.4 %) out of 142 isolates (Figure 10). Moreover, the gene *eae* + *stx2* of *E. coli* O157:H7 were detected in 18 (22.2%) out of 81 isolates (Figure 11).



**Figure 9.** Agarose gel electrophoresis of PCR products with *stx1* (110bp) primers. Lane 1: 100 bp DNA Ladder, lane 2: positive for *stx 1* genes, lane 3: Positive control, lane 4: Negative Control.



**Figure 10.** Agarose gel electrophoresis of PCR products with *stx2* (350bp) primers. Lane 1: 100 bp DNA Ladder, lane 2-6: positive for *stx 2* gene, lane 7: Positive control, lane 8: Negative Control.



**Figure 11.** Agarose gel electrophoresis of PCR products with *eae* (490 bp) primers. Lane 1: 100 bp DNA Ladder, Lane1-18: positive for *eae* gene, line 19: Positive control, line 20: Negative Control.

### 4.3. Prevalence of STEC and *E. coli* O157:H7 across milk and cottage cheese value chain

Among the four milk value chain different prevalence rate of STEC was found. The highest contamination rate was recorded in collector (23.9%) followed by producer (16.6%) and retailer (9.3%). Samples drawn from processor showed the lowest contamination rate (8.3%) (Table 8).

**Table 8.** Prevalence of STEC and *E. coli* O157:H7 across the value chain

location	Product type	Value chain	Total samples	STEC positive	<i>E. coli</i> O157:H7 positive	Prevalence %
Oromia	Raw milk	Producer	96	9	7	16/96 (16.6%)
		Collector	96	12	11	23/96 (23.9%)
	Pasteurized milk	Processor	96	8	0	8/96 (8.3%)
		Retailer	96	9	0	9/96 (9.3%)
	Cottage cheese	Producer	48	2	0	2/96 (4.1%)
		Retailer	48	0	0	

The present study revealed that STEC and *E. coli* O157:H7 were confirmed from 12% (58/480) of (milk and cottage cheese)(Table 8). The presence of *E. coli* in pasteurized milk didn't reflect the survival of the organism to the appropriate level of pasteurizing temperature. Rather, it might be due to poor hygienic handling after the milk is pasteurized, which contributes to milk contamination (Ali and Abdelgadir, 2011). In agreement with the present finding (Elhadidy and Mohammed, 2013) have show that (11.29%) of the samples were positive for STEC in Egypt. However, the prevalence is far lower when compared to the reports of (Shunda *et al.*, 2013) from Mekelle town (44%) and 23.7% by (Mekuria *et al.*, 2014) and higher than others 5.7% (Zweifel *et al.*, 2010) in Egypt and 9.23% (Yakubu *et al.*, 2018) in Nigeria. In the present study, the isolation rate of *E. coli* O157:H7 from raw milk was 22.2%, which is far higher than the prevalence report of 2.9% by (Disassa *et al.*, 2017); 12% by (Bedasa, *et al.*, 2018) and 4.08% by (Mesele and Abunna, 2019). In other study on raw milk contamination are meaningful since even one STEC in a food sample may lead to gastrointestinal or urogenital disorder due to their multiplication in the body. Therefore, strict hygiene and management practices for dairy herd and milk processing must be adopted to avoid unwanted illness (Gyles, 2007).

#### 4.4. Antimicrobial resistance profiles of STEC

In this study the *E. coli* isolates were highly sensitive to chloramphenicol (72.4%) and ciprofloxacin (75.8%). Furthermore, the majority of the isolates were resistant to ampicillin (56.8%), oxytetracycline (44.8%) and tetracycline (84.4%) (Table 9).

**Table 9.** Antimicrobial resistance profile of STEC isolated from milk and cottage cheese (n = 58)

Antibiotics	Susceptible (mm)	Intermediate (mm)	Resistance (mm)
Oxytetracycline	19 (32.7%)	13 (22.4%)	26 (44.8%)
Compound Sulphonamide	14 (24.1%)	26 (44.8%)	18 (31%)
Chloramphenicol	42 (72.4%)	9 (15.5%)	7 (12%)
Ciprofloxacin	44 (75.8%)	8 (13.7%)	6 (10.3%)
Trimethoprim	18 (31%)	21 (36.2%)	19 (32.7%)
Tetracycline	9 (15.5%)	0(0%)	49 (84.4%)
Clindamycin	18 (31%)	27 (46.5%)	13 (22.4%)
Ampicillin	16 (27.5%)	9 (15.5%)	33 (56.8%)

Source: (CLSI, 2008) mm = millimeter

The majority of the isolates were resistant to ampicillin, oxytetracycline and tetracycline similar to the previous studies from Ethiopia (Hiko *et al.*, 2008) and (Pal *et al.*, 2012) and from Botswana (Magwira *et al.*, 2005). The study revealed that the resistance of *E. coli* does exist mainly to ampicillin. Various authors reported that *E. coli* is resistant to tetracycline (Hiko *et al.*, 2008; Pal *et al.*, 2012), which is in line with this study in other investigations undertaken in Iran where resistance to tetracycline, ampicillin and trimethoprim (Zafarane *et al.*, 2017), as well as oxytetracycline and ampicillin, (Tavakoli and Pourtaghi, 2017) was the highest among STEC strains isolated from bovine with mastitis. (Zafarane *et al.*, 2017) have also reported that *E. coli* is resistant to chloramphenicol which is contrary to the results of the present study.

## **4.5. Determination of Risk factors associated with contamination of milk and cheese with STEC**

### **4.5.1. Characteristics of the study respondents**

Out of 96 milk farmers/producers in the study, majority 63(65.6%) were females. Thirty-one (32.3%) of the respondents had no any background of formal education while 31(32.3%) completed primary education. Milk production experience of the study subjects was above ten years in 50(52.1%) and two to five years in 20. The residence of 69(71.9%) of milk producers/farmers was situated in peri-urban settings (Table 11).

In the study a total of 9 milk collectors were recruited and 8 of the respondents were males. 5 of them had diploma/degree educational status while 4 had completed high school. Though most of them 4 had above 10 years of milk collecting experience 3 had 5-10 years. Moreover, the production system of 6 of milk collectors were situated in peri-urban areas (Table 11)

A total of 96 samples of pasteurized milk were collected from 7 milk processors. Out of seven milk processors 6 were males. The educational statuses of all respondent 7 were diploma and degree. Of these 5 and 1 had been engaged in milk processing for above 10 years and 2-5 years, respectively. The production system of 5 of the milk processors resided in urban dwellings while 2 of them lived in peri-urban (Table 11).

Moreover, a total of 96 pasteurized milk samples and characteristics of the Retailers were also collected from milk Retailers of Bishoftu 24, Fiche/Selale 24, Asela 24 and Wolmera 24 towns. Accordingly, 55(57.3%) were males. The educational status of 17 was diploma/degree. The remaining 29, 36(37.5%) completed high school and primary school education, respectively and 5(5.2%) respondents had no formal education. 49 (51.4%) of the retailers were in the milk market for 1-5 years. The production system of all (100%) of the milk retailers resided in urban dwellings (Table 11).

**Table 10.** Demographic characteristics of study respondents from Bishoftu, Fiche/Selale, Wolmera, and Asela towns of Oromia regional state

Variables		Raw Milk		Pasteurized milk		Cottage cheese	
		Producers/Farm ers n(%)	Collectors n (%)	Processors n(%)	Retailers n(%)	Farmers n(%)	Retailers n(%)
Sex	M	33(34.4)	8(88.9)	6(85.7)	55(57.3)	1(2.1)	9(18.8)
	F	63(65.6)	1(11.1)	1(14.3)	41(42.7)	47(97.9)	39(81.3)
	<b>Total</b>	<b>96(100)</b>	<b>9(100)</b>	<b>7(100)</b>	<b>96(100)</b>	<b>48(100)</b>	<b>48(100)</b>
Town	Bishoftu	24(25)	2(22.2)	1(14.3)	24(25)	12(25)	12(25)
	Fiche/Selale	24(25)	3(33.3)	1(14.3)	24(25)	12(25)	12(25)
	Asela	24(25)	2(22.2)	3(42.9)	24(25)	12(25)	12(25)
	Wolmera	24(25)	2(22.2)	2(28.6)	24(25)	12(25)	12(25)
	<b>Total</b>	<b>96(100)</b>	<b>9(100%)</b>	<b>7(100)</b>	<b>96(100)</b>	<b>48(100)</b>	<b>48(100)</b>
	<b>Total</b>	<b>96(100)</b>	<b>9(100)</b>	<b>7(100)</b>	<b>96(100)</b>	<b>48(100)</b>	<b>48(100)</b>
Production system	Urban	27(28.1)	3(33.3)	5(71.4)	96(100)	13(27.1)	42(87.5)
	Peri-urban	69(71.9)	6(66.7)	2(28.6)	-	35(72.9)	6(12.5)
	<b>Total</b>	<b>96(100)</b>	<b>9(100)</b>	<b>7(100)</b>	<b>96(100)</b>	<b>48(100)</b>	<b>48(100)</b>
Educational status	Diploma/Degree	9 (9.4)	5(55.6)	7(100)	17(17.7)	0	30(62.5)
	High school	18(18.8)	4(44.4)	0	29(30.2)	9(18.8)	9(18.8)
	No formal education	31(32.3)	0	0	5(5.2)	22(45.8)	2(4.2)
	Preparatory school completed	7(7.3)	0	0	9(9.4)	0	3(6.3)
	Primary school completed	31(32.3)	0	0	36(37.5)	17(35.4)	4(8.3)
	<b>Total</b>	<b>96(10%)</b>	<b>9(100%)</b>	<b>7(100%)</b>	<b>96(100%)</b>	<b>48(100)</b>	<b>48(100)</b>

Experience	≤1 year	3(3.1)	0	1(14.3)	19(19.8)	5(10.4)	7(14.6)
	1-2 years	4(4.2)	0	0	28(29.2)	9(18.8)	9(18.8)
	2-5 years	20(20.8)	2(22.2)	1(14.3)	21(21.9)	13(27.1)	24(50)
	5-10 years	19(19.8)	3(33.3)	0	13(13.5)	7(14.6)	5(10.4)
	>10 years	50(52.1)	4(44.4)	5(71.4)	15(15.6)	14(29.2)	3(6.3)
	<b>Total</b>	<b>96(100)</b>	<b>9(100)</b>	<b>7(100)</b>	<b>96(100)</b>	<b>48(100)</b>	<b>48(100)</b>

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n- number of respondents

A total of 48 cottage cheese samples and characteristics of the cottage cheese farmers/producers were collected from farm market of Bishoftu 12, Fiche/Selale 12, Asela 12 and Wolmera 12 towns. Out of the total cottage cheese farmers, 47 respondents were females and 22 had no formal education. Cottage cheese production experience of the study subjects was two to five years and above ten years in 13 and 14 of the farmers, respectively. While 35(72.9%) of the cottage cheese farmers were from peri-urban areas, the remaining were lived in urban areas, 13 (Table 11).

Finally, a total of 48 cottage cheese samples were collected and 48 Retailers were enrolled in the study. Out of 48 Retailers of Bishoftu 12, Fiche/Selale 12, Asela 12 and Wolmera 12 towns recruited in the study, 39(81.3) were females and 42(87.5%) were lived in urban areas. Of cottage cheese retailers, 30(62.5%) had diploma/degree while 2 had no formal education. The cottage cheese retailing experience of 24 of the retailers were two to five years. The retailing system of 42(87.5%) of the cottage cheese retailers were situated in urban settings (Table 11).

#### **4.6. Risk factors associated with contamination of Shiga toxin *E. coli***

##### **4.6.1. Risk Factors associated with Shiga toxin *E. coli* contamination at milk producers**

According to the findings most of the milk producers, 93 (96.88 %), wash the udder and teats of their cows. 51(53.13%) dry the washed udder, but the cleanness of the drying cloth were somewhat dirt (some soil present) in 25 (26.04%). Most positive samples for STEC were obtained from very dirty (heavily soiled) cloths 12 (12.5%) used to dry the teats and udder of the cow. 85 (88.54%) of the producers store/collect raw milk using plastic material; 57 (59.38%) of them wash milk handling equipment using tap water, 79 (82.29%) of them wash their hands before milking. The hygienic condition of the cattle barns were good in 46 (21.88) of the producers; but higher rate of STEC positive samples were recorded from barns with poor hygienic conditions which constitute 21(47.92%) (Table 12). There is a significant relationship ( $p < 0.05$ ) with the cleanness of the drying cloth and the hygienic condition of the cattle barn with positivity rate of STEC. From this the cleanness of the cattle barn was important to prevent coliform and *E. coli* in addition to Shiga Toxin *E. coli* Since low dairy house cleaning practices as well as dirty environments, in most smallholder dairy farmers, have implications on sources of pathogens (Olatunji, *et al.*, 2009; Rahel, 2008). The finding of this study is in line with the previous reports by (Bukuku, 2013; Shija, 2013; Zewdu, 2015) in Arusha, Tanga and Sidama respectively. The use of individual towel and following essential cleaning practices during milking is important for the production of quality milk. The current study is in agreement with the report of Zelalem (Zelalem, 2010).

Cleaning the udder of cows before milking is

important since it could have direct contact with the ground, urine, dung and feed refusals while resting (Zelalem, 2012). (Fufa *et al.*, 2019) reported that udder washing before milking is not widely practiced by Ethiopian dairy farmers. Of the 70 participants surveyed in their study, 26% did not wash udders prior to milking and only 30% of them used separate drying towels or cloths between milked cows to dry udders after washing. Other studies also revealed that among the participants who practiced regular washing of cows' udders, more than 80% failed to dry the washed udder using a dry and clean towel or a cloth (Abebe *et al.*, 2012; Amin *et al.*, 2017; Bekele *et al.*, 2015; Gezu *et al.*, 2015). Ethiopian farmers may use a myriad of techniques to remove dirt from udders, including allowing a calf to suckle prior to milking or using a dry cloth to remove dirt from the teats and udder of the animal. Failure to thoroughly clean and dry the udder and teats is a common source of coliforms in milk (Bekele, 2000; Pandey and Voskuil, 2011; Wubete *et al.*, 2004). Hygienic milking practices include regular cleaning and washing of animal udder and milk handling equipment before and after milking, use of separate and clean drying towels between cows, the filtering of milk after milking and avoiding the feeding of cows during milking (Muluken, *et al.*, 2020).

Maintaining the sanitary condition of milking area is important prerequisite for clean milk production (Zelalem, 2010). Unhygienic milking environments can facilitate the spread of microorganisms (Fuentes *et al.*, 2014; Zdanowicz *et al.*, 2004). Exposure of cow's udder to environment contaminated with feces or debris is a major source of microbial contamination of milk (Abunna *et al.*, 2019; Vacheyrou *et al.*, 2011). Additionally, irregular cleaning of the milking areas and animal sheds contributes to cross-contamination of milk in household dairy farms (Carloni *et al.*, 2016). This is a major challenge in Ethiopia, as on-farm infrastructure is commonly underdeveloped. And most of the cow sheds are built using trees while a few of them are made of blocks and iron sheets (Shija, 2013). Milking is conducted inside a confined shed on a majority of smallholder dairy farms in Ethiopia, where there is a high risk of contamination through the dusty air and insects (Abebe *et al.*, 2012). Lack of sufficient space, especially in urban areas, and irregular cleaning of milking rooms and cowsheds can create suitable conditions for the growth of insects like flies that can transmit pathogens (Pandey and Voskuil, 2011).

**Table 11.** Factors associated with contamination of Producers raw milk by *Shiga toxin E. coli*

Conditions	Risk factors	Animals examine d	Positives	%	95% CI	OR	X <sup>2</sup>	p- Value
<b>Washing the udder and Teats</b>	Yes	94	16	17.0	7.2-16.2	-	-	<b>0.310</b>
	No	2	0	0	-	-		
<b>Drying the washed udder</b>	Yes	51	9	17.6	9.4-30.6	1.2	0.08	<b>0.723</b>
	No	45	7	15.6	3.0-52.7	1		
<b>Cleanness of the drying cloth</b>	Visibly clean	13	2	15.4	3.9-45.1	1	11.22	<b>0.032*</b>
	Somewhat dirty	25	5	20.0	0.9-87.2	1.4		
	Very dirty	13	9	69.2	6.9-98.6	12.4		
<b>Milk Handling using</b>	Plastic	85	14	16.5	10.0-25.9	1	0.94	<b>0.544</b>
	Aluminum cans	6	2	33.3	4.5-84.2	2.5		
<b>Water source to wash milking equipment</b>	Mazzi plastic	5	0	0	0	-		<b>0.435</b>
	Ground water	9	2	22.2	5.6-57.9	2.0	2.33	
	Pump water	23	6	26.1	1.2-91.3	2.5		
	Rain water	1	1	100.0	-			
	River water	6	0	0.0	-			
<b>Washing hands before milking</b>	Tap water	57	7	12.3	0.5-79.7	1		<b>0.544</b>
	Yes	79	15	19.0	11.8-29.1	3.8	2.11	
<b>Hygienic Condition of barn</b>	No	17	1	5.9	0.4-47.2	1		<b>0.045*</b>
	Good	46	0	0	-	-	13.85	
	Medium	28	3	10.7	1.2-54.9	12		
	Poor	22	13	59.1	38.2-77.2	1		

n- Number of milk producers

\*- Significant association

N- STEC PCR positive samples

#### **4.6.2. Risk factors associated with Shiga toxin *E. coli* contamination at milk collectors**

About 6 (66.67%) of the milk collectors have a cooling system to preserve their milk. (55.56) of the hygiene conditions of the collection centers were medium (partially concrete floor, irregular cleaning, insufficient ventilation, inadequate lighting), the floor in milk collection and/or storage area was free of trash 6 (66.67%), but higher PCR positive samples were recorded. The floor in milk collection and/or storage area wet with accumulated dirty water 5 (55.56%). There was (faces, hair etc.) in the collection area 3 (33.33%). The collector milk was gathered using aluminium cans 8(77.8%) of the cases (Table 13). There is a significant relationship with the hygienic condition of the collection center and the presence of trash, faces, hair etc in the collection area and PCR-positivity rate of STEC (P-value <0.05) (Table 13). Maintaining the sanitary condition of milking and collecting area is important for the production of good quality milk (Zelalem, 2010). Dirty environment and lack of cold storage facilities were together regarded as main risk factors that contributed to the high bacterial contamination of the milk Kivaria *et al.* (2006). These findings are in line with the current study. According to (Tigabu *et al.*, 2015) poor storage and transportation conditions can further facilitate the contamination of milk from milk handling equipment. Since, Ninety percent of the milk produced by smallholders is marketed in an informal marketing system; and only the remaining 10% is delivered to the formal market (SNV, 2008). The hygienic conditions of the informal markets are not monitored or sustainably maintained (SNV, 2008; Mohamed *et al.*, 2004; Kebede *et al.*, 2019; Tsehay, 2001; Tsadkan and Gurja, 2018).

**Table 12.** Factors associated with contamination of collection center raw milk by *Shiga toxin E.coli*

Conditions	Risk factors	Animals examined	Positives	%	95% CI	OR	X <sup>2</sup>	p-Value
<b>Do you have a cooling system to preserve your milk?</b>	Yes	79	15	19.0	11.8-29.1	3.8	2.11	<b>0.077</b>
	No	17	5	20.0	0.9-87.2	1.4		
<b>Hygienic condition of the collection center</b>	Good	9	2	22.2	5.6-57.9	2.0	2.33	<b>&lt;0.01*</b>
	Medium	64	12	12.5	7.2-20.7	96		
	Poor	23	6	26.1	1.2-91.3			
<b>The floor in milk collection and/or storage area free of trash</b>	Yes	39	13	59.1	38.2-77.2	1	22	<b>0.001*</b>
	No	57	7	12.3	0.5-79.7	1		
<b>The floor in milk collection and/or storage area wet with accumulated dirty water</b>	Yes	51	9	17.6	9.4-30.6	1.2	0.08	<b>0.185</b>
	No	45	11	11.5	6.5-19.5	11		
<b>The present of (feces, hair etc.) in the collection area</b>	Yes	28	3	10.7	1.2-54.9	12	28	<b>0.047*</b>
	No	68	6	26.1	1.2-91.3	2.5		
<b>Milk collecting Equipment</b>	Aluminum can	79	15	19.0	11.8-29.1	3.8	2.11	<b>0.490</b>
	Plastic	17	5	20.0	0.9-87.2	1.4		

n- Number of milk collectors

\*- Significant association

N- STEC PCR positive samples

#### **4.6.3. Risk Factors associated with Shiga toxin *E. coli* contamination at milk processors**

As observed during the current study, 5(71.4%) of the processors use four-wheel drive for transportation of processed milk and their milk suppliers were individual farms 6(85.7%) big farms 4(57.14%) and milk collectors 2 (28.57%). Mostly the processors don't maintain the temperature during transportation 4 (57.14%) in relation to this higher number of PCR positive sample were detected. Most of them 5 (71.43%) use tap water for washing milk handling equipment and they calibrate their system 6 (85.71%). In this study, significant associations were observed between STEC positive samples and milk suppliers and maintaining the temperature during milk transportation with a p-value <0.05 (Table 14). Storage, handling and transportation of milk under room temperature increases bacteria multiplication, since the milk is managed at an ambient temperature, high microbial populations can be reached within short period of time (Zelalem, 2010). Temperature of storage is an important factor in determining milk quality as this influences the rate at which the bacteria will increase in number (Lore *et al.*, 2006). Raw milk can only be kept for hours without storage at an appropriate temperature (4°C) before it deteriorates in both quality and safety (SNV, 2008). Therefore, it must be stored and kept cool using proper refrigeration within two hours after milking, it maintains nearly its original quality and remains fresh for a reasonably longer time until processing and consumption (Pauline and Karin, 2006; SNV, 2008) However, such storage facilities are not readily available in Ethiopia, particularly in rural areas and cooling systems are not feasible due to lack of the required dairy infrastructure and unstable power supply (Eshetu *et al.*, 2019; O'Connell *et al.*, 2016). In conclusion, milk should be cooled to a suitable temperature (4°C) and transported by means that maintain its quality and safety (Muluken *et al.*, 2020). In this study more STEC prevalence were observed on the Individual farms, since most dairy farm owners in the present study do not sufficiently perform cleaning of cow's udder and teat with potable water and do not dry it properly this may probably increase microbial contamination of milk. Similar reports were done by (Swai and Schoonman, 2011) and (Shija, 2013) in the Tanga region of Tanzania.

**Table 13.** Factors associated with contamination of processors pasteurized milk by *Shiga toxin E. coli*

Conditions	Risk factors	Animals examined	Positives	%	95% CI	OR	X <sup>2</sup>	p-Value
<b>Transportation Mechanisms</b>	Four-wheel drive	50	6	26.1	1.2-91.3	2.5	23	<b>0.302</b>
	Public transport	23	1	100.0	-			
<b>Milk suppliers</b>	Cool transport	23	1	100.0	-			<b>0.05*</b>
	Individual farms	2	5	20.0	0.9-87.2	1.4	25	
	Big dairy farms	5	2	22.2	5.6-57.9	2.0		
	Milk collectors	1	1	5.9	0.4-47.2	1		
<b>Do you maintain the Temperature during transportation</b>	Yes	73	2	22.2	5.6-57.9	2.0	9	<b>0.002*</b>
	No	23	6	26.1	1.2-91.3	2.5		
<b>Source of water for washing milk handling equipment</b>	Tap water	2	5	20.0	0.9-87.2	1.4	25	<b>0.248</b>
	Ground water	7	2	22.2	5.6-57.9	2.0		
<b>Do you calibrate yoursystem</b>	Yes	1	6	26.1	1.2-91.3	2.5	25	<b>0.131</b>
	No	69	5	20.0	0.9-87.2	1.4		
		27						

n- Number of milk processors

\*- Significant association

N- STEC PCR positive samples

#### 4.6.4. Risk Factors associated with Shiga toxin *E. coli* contamination at milk retailer's

Mostly the time to transport pasteurized milk to retail shops was less than 30 min 7 (7.22%), but higher number of STEC positive samples were recorded in the time over 1 hr 6 (6.19%) (Table 15). Majority of the retailers 78(80.41%) had no separate refrigerator for storing of the milk until sell had no backup generator to be used in case when electric power fails 77 (79.38%) and they never took training related to good milking and personal hygiene practices for handling milk 92(94.85%) (Table 15). There were significant relationships with the time to transport pasteurized milk to retail shop with the STEC positivity (P-value < 0.05). when the time of the pasteurized milk transport to retail shop was delayed the count of bacteria was increased. The time it takes to transport or deliver milk to different value chains has been reported as a factor that affects its quality and safety (Muluken *et al.*, 2020) According to (Tigabu *et al.*, 2015), samples from dairy farmers that had more than a 30 min travel time to the collection center had a 5.6 times higher risk of contamination with *Staphylococcus aureus* when compared to farmers that had less than 30 min of travel time to the collection centers.

**Table 14.** Factors associated with contamination of retailer's pasteurized milk by *Shiga toxin E. coli*

Conditions	Risk factors	Sample examined	Positives	%	95% CI	OR	X <sup>2</sup>	p-Value
<b>Time to transport pasteurized refrigerator to store</b>	< 30 Min	47	3	6.4	2.1-18.0	1.6	10.37	
	½ hr – 1 hr	24	1	4.2	0.1-58.7	1		<b>0.015*</b>
	Over 1 hr	25	8	32.0	3.3-86.5	10.8		
<b>Backup generator</b>	Yes	18	2	11.1	2.8-35.2	1	0.04	<b>0.178</b>
	No	78	10	12.8	0.7-76.2	1.2		
<b>Training to good milking</b>	Yes	4	1	25.0	3.4-76.2	2.5	0.49	<b>0.537</b>
	No	92	11	12.0	0.1-93.2	1.0		

n- Number of milk retailers

\*- Significant association

N- STEC PCR positive samples

#### **4.6.5. Risk Factors associated with Shiga toxin *E. coli* contamination at cottage cheese producers**

In this study 40 (83.33%) of the cottage cheese producers used plastic buckets as a packing materials to handle the cottage cheese, and about 40 (83.33%) of them were poor quality with a significance relation with the PCR positive sample. Mostly they wash cottage cheese handling equipment and utensils before and after use 47 (97.92%) by water with soap/ detergents, 31 (64.58%) most respondents never took training related to safety and quality of cottage cheese handling 44 (91.67%) From 48 samples collected from the cottage cheese producers, STEC was recorded, and there was a significant relationship with the quality of packaging materials and STEC positivity (Table 16). Packaging materials of cottage cheese determine the quality of milk products. Producers need therefore pay particular attention for the type as well as cleanliness of the materials. (Zelalem, 2012). Informal marketing systems are widely observed in traditional open markets and at the household level, in which limitations on infrastructure, proper packaging, storage and transportation equipment are present (Asresie *et al.*, 2018; Seifu and Tassew, 2014). The hygienic conditions of the informal markets are not monitored or sustainably maintained (SNV, 2008; Mohamed *et al.*, 2004; Kebede *et al.*, 2019; Tsehay, 2001; Tsadkan and Gurja, 2018). According to the Central Statistics Agency (CSA), of the total urbanmilk production, 73% is sold, 10% is left for household consumption, 9.4% goes to calves and 7.6% is processed into butter and cheese (CSA, 2011).

**Table 15.** Risk factors associated with producer cottage cheese and Shiga toxin *E. coli*

<b>Conditions</b>	<b>Risk factors</b>	<b>Sample examined</b>	<b>Positives</b>	<b>%</b>	<b>95% CI</b>	<b>OR</b>	<b>X<sup>2</sup></b>	<b>p-Value</b>
<b>Packaging material for cottage cheese handling</b>	House paint Buckets	15	0	0	-	-	13.85	<b>0.135</b>
	Metal can	14	0	0	-	-		
	Plastic bucket	19	2	10.5	2.6-33.7	1	0.09	
<b>The quality of packaging materials</b>	Good	29	0	0	-	-	14	<b>0.046*</b>
	Poor	19	2	10.5	2.6-33.7	1		
<b>Washing cottage cheese handling equipment and utensils before and after use</b>	Yes	34	2	10.5	2.6-33.7	1	19	<b>0.248</b>
	No	14	0	0	-	-		
<b>Packaging material for cottage cheese handling</b>	Water with soap/detergents	17	1	5.9	0.4-47.2	1		<b>0.368</b>
	Different herbs	14	0	0	-	-		
	Cold water only	17	1	5.9	0.4-47.2	1		
<b>Training related to safety and quality of cottage cheese Handling</b>	Yes	21	0	0	-	-	19	<b>0.248</b>
	No	27	2	10.5	2.6-33.7	1		

n- Number of cottage cheese producers \*-Significant association N- STEC PCR positive samples

#### **4.6.6. Risk Factors associated with Shiga toxin *E. coli* contamination at cottage cheese retailer's**

Out of 48 cottage cheese samples collected from retailers none of them had positive for *Shiga toxin E. coli* STEC. Although cottage cheese (ayib), due to its low pH value, does not seem to play a significant role in the transmission of food-borne diseases, its keeping quality may be reduced by its load of acid-tolerant microorganisms (Ashenafi, 1990).

## 5. Conclusions and Recommendation

The outcome of this study showed that, of raw milk, of pasturized milk and of cottage cheese were higher than that recommended by Ethiopian and East African Standards which implies the microbial qualities were poor, as judged from the high values of total bacterial count (TBC), total coliform count (TCC) and *E. coli* count.

The counts of TBC, TCC and *E. coli* had significant difference along the milk value chain. The raw milk samples collected from producers and collectors had high TBC and TCC. However, *E. coli* had high counts at producers and retailer's value chain followed by collectors and processors that can be related to the poor hygienic managements. In addition TBC, TCC and *E. coli* counts of cottage cheese samples did not show statistically significant difference between the producer and retail.

The findings of this study revealed that raw milk; pasteurized milk and cottage cheese contamination with STEC in high milk producing areas of Ethiopia indicating a significant public health hazard. The contamination status of the raw milk, pasteurized milk and cottage cheese samples was considerably different with highest proportion of STEC in raw milk samples. Among the value chains the largest contamination of STEC positivity was detected from collectors mainly due to the hygienic condition of the collection center which was poor. Most STEC positive producer cottage cheese samples come from the poor quality of packaging materials used for handling.

In general, the presence of these pathogens could be attributed to the poor sanitary conditions of person in contact with the milk and its products, poor storage and transportation mechanisms/equipment, unclean milk and cheese storage equipment, poor handling mechanism and failure in maintaining storage temperature, lack of cool transportation and distribution systems also contributes to the spoilage of milk and milk products.

Most of the STEC and *E. coli* O157:H7 isolates were subjected to antibiotic sensitivity test showed different degrees of resistance against the antibiotic discs tested, including ampicillin, tetracycline and oxytetracycline. This is an indication that there is a need for extensive study on the occurrence, risk factors and genetic back ground of antimicrobial resistance of STEC and *E. coli* O157:H7 in the study area.

As a number of people in Ethiopia consider raw milk as safe and consumed it without further processing, there is a higher possibility that high risk group will consume contaminated milk and milk products and may contract severe illness and hemolytic uremic syndrome. Particularly, the highest presence of STEC and *E. coli* O157 H7 in raw milk poses public health threat to consumers especially for the very young children, the elder and immune-compromised individuals.

Based on the findings from this study the following recommendations are forwarded:

- Proper milking management methods such as pre milking udder preparation, proper removal of the milk and post milking teat dipping should be introduced and promoted.
- Efficient milk cooling system at affordable prices is required along the value chain. Since there is a long time interval between milking and delivery.
- Sustainable awareness should be created on the hygienic milk production and handling practice along the value chain.
- Establishing milk processing plants around high milk producing areas, could contribute towards reducing further contamination of milk due to time elapsed during transportation.
- Training should also be given to milk processors on Good Manufacturing Practices and Hazard Analysis Critical Control Point (HACCP) so as to identify and avoid contamination of milk and milk products.
- Actors in each critical point should perform basic laboratory tests for at least indicator microorganisms.
- Regulatory bodies should conduct sustainable monitoring activities along the value chain.
- Other diarrheagenic pathogen and virulence genes of *E. coli* should be investigated.
- Further studies should be recommended to verify the uses of Petrifim for *E. coli* O157:H7.
- Use active drugs like Chloramphenicol and Ciprofloxacin is recommended in controlling *E. coli* infections in dairy cows. In addition, Regular antimicrobial susceptibility surveillance should be accustomed.

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## 7. Annexes

### Annexes 1: Consent form

Dear respondent, good morning/good afternoon. Thank you for your interest in talking with me today. I am Betelhem Mengstu from Addis Ababa University. I am conducting a study on The Prevalence, Antimicrobial Resistance Profiles and Assessment of the risk factors Associated with Shiga Toxin Producing Escherichia coli (STEC) in milk and cottage cheese across the dairy value chain in Oromia regional state, Ethiopia. The purpose of my visit today is to collect information from you on the aforementioned issue. This study will be conducted in selected towns (Fiche/Selale, Wolmera, Deber-zeit (Bishoftu) and Asela) of Oromia Regional State of Ethiopia. If you are willing to participate in the study, I will ask you few questions for 20-30 minutes. Your name will be confidential and the data will never be used in disclosing your information. You do not have to answer any question that you are not comfortable with, and you may end this task any time you want to. However, your honest answers to these questions will help us in better understanding of the safety of milk and dairy products and will eventually help in designing and implementing appropriate interventions to alleviate related problems. Hence, I greatly appreciate your participation in the study.

### Annexes 2: Questionnaire to be filled by Milk producers/ farmers

<b>Are you willing to participate in the study?</b>
A. Yes      B. No

#### Section A: Preliminary Information

1. Name of Interviewer ----- date ----- Time----- Respondent code no. -----  
-

#### 2. Geography and location

- **Town/District/ Zone ----- Woreda/ Kebele ----- Status:** A. Urban B. Peri-Urban (Peri-urban: areas that are located near to cities or large urban areas but retain rural characteristics such as substantial reliance on agriculture)
- **current location** Latitude (x.y °) ----- Longitude (x.y °)-----  
Altitude (m) .....

#### Section B: Milk handling knowledge gap assessment of the respondent

4. **How long have you been engaged in milk production?** In case of farm/ union/ cooperatives: How long it's been the farm engaged in milk production?      A. Less than one year      B. 1-2 years      C. 2-5 years

➤ 5-10 years

E. Over 10 years

**4. Farm conditions (infrastructure, hygiene and animal Health)**

**5. Observational: What does the milking house look like?** A. Kraal B. Cattle shed

C. Concrete floor barn

D. Soil floor barn

E. Other, specify

**5. Observational: what is the hygienic condition of the cattle barn look like?**

A. Poor (soil and dirt floor, irregular cleaning, no feeding stand,)

B. Medium (partially concrete floor, irregular cleaning, old and wood feeding stand,)

C. Good (concrete/cement floor, daily cleaning, separate concrete feeding stand,)

**6. Observational: Is there animal manure (remnant of dung/feces) of the animal present in milking area?**

1. Yes B. No

**7. Observational: Are there live pests (rodents or insects) or any sign of it (feces, hair etc.) present in the milking area?** A. Yes B. No

**8. What is your major source of water for washing milk and cottage cheese handling equipment?**

A. Ground water

B. Pump water

C. River water

D. Rain water E. Tap

water

• **Milking Conditions (gender role, personal hygiene, milking equipment and milking time)**

**5.1: Gender role in milking and milking time**

**1) Who does the milking?** A. Mostly male adult task (Husband) B. Mostly female adult task (Wife)

C. Mostly male child task (Son)

D. Mostly female child task (Daughter)

E. Employees

F. Members of the union/cooperative

G. Relatives

**5.2: Personal hygiene and sanitation**

**4. Observational: Is the milker wearing a head cover or a hairnet during milk**

**handling?** (Ask the respondent if you did not get the chance to observe) A. Yes

B. No

**5. Observational: Does the milker wash their hands before milking and milk handling?**

(Ask the respondent if you did not get the chance to observe)

A. Yes B. No

**6. Observational: Does the milker wash their hands after touching their face, clothing**

**and cows?** (Ask the respondent if you did not get the chance to observe) A. Yes B. No

**7. Do you cut your nails regularly? (at least twice a week)** (Ask the respondent if you did

not got the chance to observe)

A. Yes B. No

### 5.3. The Cow

1. Do you wash the udder and teats of the cows before milking? A. Yes B. No

2. Do you dry the washed udder and teats by using dry cloth? A. Yes B. No

3. Observational: What does the cleanliness of the drying cloth looks like? A. Yes B. No

### 5.4: Milking Equipment

1. What type of milk handling equipment or container do you use?

A. Mazzi plastic container B. Plastic containers C. Aluminum cans D. Others \_\_\_\_\_

2. Do you wash the milking equipment and utensils before and after milking?

A. Yes B. No

3. Observational: How is the milking equipment stored after milking?

A. Upright and open

B. Upright but covered

C. Upside down in contact with the ground

D. Upside down on shelf

6. Post Milking Conditions (Storage, Cooling, Transportation, Marketing and Consumption)

#### 6.1: Storage and Cooling of milk

1. Observational: Do you have a refrigerator as a cooling mechanism to store or preserve milk at household level until sale or consumption? A. Yes (if yes Ask to show you) B. No

2. Do you use other preserving mechanism other than cooling in a refrigerator to store or preserve milk at household until sale or consumption? A. Yes B. No

• What do you use as a preserving mechanism other than cooling in a refrigerator to store or preserve milk at household until sale or consumption?

4. Using artificial chemicals (such as formalin farmer call it milk medicine)

5. Using natural herbs or chemicals

6. Concentrating it to powder

7. Fermentation or processing to other milk products (Yogurt, cheese or butter) E. Other

#### 6.2. Transportation and marketing of milk

1. Do you sell milk to others? A. Yes B. No

2. What do they use as a means of transportation for delivering milk to the consumer/ other?

• Motor bicycles B. Three wheel drive "Bajaj" C. Animal drawn cart

#### 6.3. Consumption of milk at household level

1. Do you or anyone from your family consume raw milk at home? A. Yes B. No
2. Do you have the awareness about bacteria that might get you sick if you consume raw milk?  
A. Yes B. No
3. Have you or anyone from your family ever faced any illness associated with raw milk consumption? A. Yes B. No
4. Do you boil milk before consumption? A. Yes B. No
5. Have you or anyone from your family ever faced any illness associated with boiled milk consumption? A. Yes B. No

**Additional comments from the respondent about milk production job.**

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Put any comment the respondent gives here about what kind of support the farmer need or any other things related to milk production job

*Thank You for your time!!!*

**Annexes 3: Questionnaire to be filled by Milk collectors**

<b>Are you willing to participate in the study?</b>
---

A. Yes      B. No
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**Section A: Preliminary Information**

1. Name of Interviewer ----- date ----- Time----- Collection center code. -----

**2. Geography and location**

- **Town/District/ Zone ----- Woreda/ Kebele ----- Status:** A. Urban B. Peri-Urban (Peri-urban: areas that are located near to cities or large urban areas but retain rural characteristics such as substantial reliance on agriculture)
- **current location** Latitude (x.y °) ----- Longitude (x.y °)-----  
Altitude (m) .....

**Section B: Personal details of the respondent representing the retail shop**

1. **Gender of the respondent** A. Male B. Female
2. **Position of the respondent in the shop:** A. Owner B. Employee C. Relative **Other** \_\_\_\_\_
3. **Educational status of the respondent**

A. No formal education B. 1-8 B. 9-10 C. Preparatory school D. Diploma/Degree

**Section C: Transportation of milk from farm to collection center (milk collection and transportation system, milk cooling and storage)**

**1. What do they use as a means of transportation for delivering milk to the center?**

A. Trekking on foot B. Three wheel drive "Bajaj" C. Animal drawn cart D. Refrigerated vehicles/ cold chain E. Own transport vehicle F. Public transport G. Other, specify

**2. Observational: What does the Personnel hygiene of milk transporters (drivers) look like?**

(Ask the respondent if you did not got the chance to observe)

A. Poor (Very dirty) B. Medium (Dirty) C. Good (Clean)

**3. Do you filter milk up on reception? A. Yes B. No**

**4) What do you use for filtration? A. Piece of cloth B. Plastic filter**

**Section C-2: Milk cooling and storage conditions at the centre**

**1) Do you have a cooling system to preserve your milk? A. Yes B. No**

**5) What do you use as a cooling system to preserve milk?**

A. Refrigerator set at 4°C B. Deep freezer/s C. Bulk tankers

**Section D: Collection center conditions (infrastructure, milk handling equipment and personal hygiene)**

**» Section D-1: Infrastructure and milk handling equipment**

**1) Observational: What does the storage and/or collection house look like?**

A. Soil floor storage room B. Concrete floor storage room

**2) Observational: What is the hygiene condition of the collection center look like?**

A. Poor (soil and dirt floor, irregular cleaning, no ventilation, in adequate lighting,...)  
B. Medium (partially concrete floor, irregular cleaning, insufficient ventilation, ...)  
C. Good (concrete/cement floor, regular cleaning, sufficient ventilation, adequate lighting,...)

**3) Observational: Is the floor in milk collection and/or storage area wet with accumulated dirty water (washing/cleaning water)? A. Yes B. No**

**4. Observational: Are there live pests (rodents or insects) or any sign of it (feces, hair etc.) in the collection/storage room? A. Yes B. No**

**5. Observational: Is the floor in milk collection and/or storage area free of trash? A. Yes B. No**

**6) What is the major source of water for washing milk handling equipment?**

A. Ground water B. Pump water C. River water D. Rain water

**7) What type of milk handling equipment or container do you use?**

A. Plastic containers B. Aluminum cans C. Mazzi cans

**Section D-2: Personal Hygiene**

**1) Observational: Is the milk handler wearing a head cover or a hairnet during milk handling?** (Ask the respondent if you did not get the chance to observe) A. Yes B. No

**2) Observational: Does the milk handler wear any gloves to handle milk?** (Ask the respondent if you did not get the chance to observe) A. Yes B. No

**Section E: Marketing and transportation to the consumers**

**1) Do you transport milk to the buyers by using your own transportation?** A. Yes B. No

**2) What do you use as a means of transportation for delivering milk to buyers?**

A. Insulated bulk tanks/cold chain B. Hoarse carts C. Three wheel drive "Bajaj" D. Four wheel drive without insulated bulk tankers E. Trekking on foot F. Public transport

*Thank you for your time!!!*

**Annexes 4: Questionnaire to be filled by Milk processors**

**Are you willing to participate in the study?**

A. Yes B. No

**Section A: Preliminary Information**

1. Name of Interviewer ----- date ----- Time----- Processing factory code no. -----

-

**2. Geography and location**

➤ **Town/District/ Zone ----- Woreda/ Kebele ----- Status:** A. Urban B. Peri-Urban (Peri-urban: areas that are located near to cities or large urban areas but retain rural characteristics such as substantial reliance on agriculture)

➤ **current location** Latitude (x.y °) ----- Longitude (x.y °)-----  
Altitude (m) .....

**Section B: Personal details of the respondent representing the retail shop**

**1. Name of Milk processor \_\_\_\_\_ Respondent name \_\_\_\_\_** (Confidential)

**2. Gender of the respondent** A. Male B. Female

**3. Position of the respondent in the shop:** A. Owner B. Employee C. Relative **Other \_\_\_\_\_**

**4. Educational status of the respondent**

➤ A. No formal education B. 1-8 B. 9-10 C. Preparatory school D. Diploma/Degree

**Section C: Milk collection and transportation conditions to the processing plant**

**1. Who are your milk suppliers?** A. Individual farms B. Big dairy farms C. Milk collectors

**2. What do you use as a means of transportation for delivering milk to the processing plant?**

A. Motor bicycles B. Three wheel drive "Bajaj" C. Four wheel drive

- D. Trekking on foot            E. Public transport

**Section D: Milk reception and pre-pasteurization conditions at the processing plant (milk quality test at reception point, hygienic condition of the processing facility and personnel)**

» **Section D-1: Milk quality test at the reception point**

1. **Do you check the quality of milk during reception?** A. Yes B. No
2. **How often do you check or test the quality of milk?** A. Regularly    B. Sometimes
3. **Do you filter milk before processing?** A. Yes            B. No

**Section D-2: Hygienic condition of the processing facility, milk handling equipment and personnel)**

1. **Observational: What is the hygiene condition of the processing plant look like?**  
A. Poor (soil and dirt floor, irregular cleaning, no ventilation, in adequate lighting,...)  
B. Good (concrete/cement floor, regular cleaning, sufficient ventilation, adequate lighting,...)
2. **Observational: What is the source of water for washing milk handling equipment?**  
A. Tap water    B. Ground water    C. Pump water            D. River water

**Section E: Pasteurization processing technology and hygienic conditions of the pasteurization process**

1. **What is the holding time and temperature combination you used for pasteurization?**  
A. LTLT (low temperature long time) (63 °C for 30 minutes)  
B. HTST (High temperature short time) (72 °C for 15 seconds)  
C. UHT (Ultra high temperature) (135°C for 2-4°C)  
D. Flash Pasteurization
2. **Do you calibrate your system to assure that appropriate temperature and time is maintained in the process line during pasteurization?**            A. Yes            B. No

**Section F: Post pasteurization conditions (storage, packaging and storage conditions)**

1. **Do you have cold storage to store the pasteurized milk before dispatch?** A. Yes    B. No
2. **Do you have a cold chain transportation system to maintain the temperature of the pasteurized milk?** A. Yes            B. No
3. **What is the temperature of the cold storage?** A. 4 °C    B. -8 °C
4. **Observational: What is the packaging material in which pasteurized milk is packed?**  
A. Paper board packages    B. Plastic pouches            C. Plastic bottles C. Plastic pouches
5. **What do you label on the packaging material?**  
A. Expiration date            B. Batch number            C. Brand name

**Additional comments from the respondent about milk processing job**

**Put any comment the respondent gives here about what kind of support the processor need or any other things related to milk processing job**

---

*Thank you for your time!!!*

**Annexes 5: Questionnaire to be filled by Milk Retailers**

<b>Are you willing to participate in the study?</b>
A. Yes      B. No

**Section A: Preliminary Information**

**1. Name of Interviewer ----- date ----- Time----- Retail shop code No . -----**

**2. Geography and location**

- **Town/District/ Zone ----- Woreda/ Kebele ----- Status:** A. Urban B. Peri-Urban (Peri-urban: areas that are located near to cities or large urban areas but retain rural characteristics such as substantial reliance on agriculture)
- **current location** Latitude (x.y °) ----- Longitude (x.y °)-----  
Altitude (m) .....

**Section B: Personal details of the respondent representing the retail shop**

- 1. Respondent name \_\_\_\_\_(Confidential)**
- 2. Gender of the respondent** A. Male      B. Female
- 3. Position of the respondent in the shop:** A. Owner    B. Employee    C. Relative    **Other \_\_\_\_\_**
- 4. Educational status of the respondent**
  - A. No formal education    B. 1-8      B. 9-10    C. Preparatory school    D. Diploma/Degree
- 3. Have you or anyone from the shop attended any training related to safety and quality of milk handling?**    A. Yes                      B. No

**Section c: packaging material and quality test of pasteurized milk**

- 1. What do they use as a means of transportation for delivering milk to the retail shop?**
  - Motor bicycles      B. Three wheel drive "Bajaj"      C. Animal drawn cart
- 2. Observational: What is the packaging material in which pasteurized milk is received from processors?** A. Paper board packages      B. Plastic bottles      C. Plastic pouches

**Section D: Storage conditions at the retail level**

**1. Observational: What do you use to maintain the temperature of the pasteurized milk until sell?**

- A. Refrigerator      B. Deep freezer/s      C. Bulk tankers      D. Cold water

**2. Observational: Is your refrigerator temperature set at 4 -8°C to store pasteurized milk until sell?** (please check the temperature of the refrigerator by using thermometer if they allow you to do that) A. Yes B. No

**3. Observational: Do you have backup generator to be used in case when electric power is out?**      A. Yes      B. No

**4. Observational: Do you have a separate refrigerator to store pasteurized milk until sell?**  
A. Yes      B. No

**5. How long does it take to transport pasteurized milk from processing facility to retail shop?**

- A. Less than 30 min      B. Over 1 hr      C. ½ hr – 1 hr

**Section E: Storage conditions at the retail level**

**1. Did you ever faced rejection of your pasteurized milk by buyers?** A. Yes B. No

**2. How often do you face?**      A. Rarely      B. Mostly      C. Sometimes

**3. Do you know their reason for rejection?**      A. Yes B. No

**4. What is their reason for rejection?** A. Expiration of milk      B. Color

**Additional comments from the respondent about milk production job**

**Put any comment the respondent gives here about what kind of support the farmer need or any other things related to milk production job**

---

*Thank you for your time!!!*

**Annexes 6: Questionnaire to be filled by Questionnaire for Cottage cheese producers**

**Are you willing to participate in the study?**

- A.Yes      B. No

**Section A: Preliminary Information**

1. Name of Interviewer ----- date ----- Time----- Respondent code no.-----

--

**2. Geography and location**

➤ **Town/District/ Zone ----- Woreda/ Kebele ----- Status:** A. Urban B. Peri-Urban (Peri-urban: areas that are located near to cities or large urban areas but retain rural characteristics such as substantial reliance on agriculture)

➤ **current location** Latitude (x.y °) ----- Longitude (x.y °) -----  
Altitude (m) .....

### 3. Processing and Storage of cottage cheese

- **Do you make cottage cheese at home?** A. Yes B. No
- **Observational: How do you preserve cottage cheese in a household until sale or consumption?**

A. Placing underground    B. Placing under water    C. Refrigerator    D. **Other, Specify**

B. **Have you ever attended any training related to cottage cheese handling practices?**    A. Yes    B. No

**4. Observational: Is there a refrigerator to store /preserve cottage cheese at household level until sale or consumption?**

A. Yes    B. No

**Section B: Hygienic condition of cottage cheese handling equipment and packaging material:** A. Good    B. Poor

**6. Do you regularly wash cottage cheese handling equipment and utensils before and after use?** A. Yes B. No

**7. What kind of cleaning chemical do you use for washing and cleaning of cottage cheese handling equipment and utensils?** A. Soap and detergents    B. Cold water only    **Other, Specify**

-----

**8. Observational: What type of packaging material do you use to handle cottage cheese?**

A. Plastic cans    B. Polyethylene bags "festal"

### 9. Transporting, sells and marketing of cottage cheese

**1. How is cottage cheese transported to the farm markets/ retail shops/hotels?**

1. Trekking on foot    B. Three-wheel drive "Bajaj"    C. Animal drawn cart

i. Refrigerated vehicles/ cold chain    E. Own transport vehicle    F. Public transport

**2. How long does it take to transport cottage cheese to farm market/retail shop/hotels?**

A. Less than 30 min    B. ½ hr. – 1 hr.    C. Over 1 hr.

**10. Consumption of Cottage Cheese at Household Level**

- 3. Do you or anyone from your family consume cottage cheese at home?    A. Yes  
B. No
- 4. Do/did you ever face any illness associated with consumption of cottage cheese?    A.  
Yes    B. No
- 5. What type of illness is/was that?    A. Diarrhea    B. Abdominal pain  
C. Typhoid    D. Vomiting

**11. Additional comments from the respondent about milk production job**

Put any comment the respondent gives here about what kind of support the farmer/producer need or any other things related to cottage cheese production job

*Thank you for your time!!!*

**Annexes 7: Questionnaire to be filled by Cottage Cheese Retailers**

<b>Are you willing to participate in the study?</b>
A. Yes                      B. No

**Section A: Preliminary Information**

1. Name of Interviewer ----- date ----- Time-----    Retailer's code no. -----  
-

**2. Geography and location**

- **Town/District/ Zone ----- Woreda/ Kebele ----- Status:** A. Urban B. Peri-Urban (Peri-urban: areas that are located near to cities or large urban areas but retain rural characteristics such as substantial reliance on agriculture)
- **current location** Latitude (x.y °) ----- Longitude (x.y °) -----  
Altitude (m) .....

**Section B: Personal details of the respondent representing the retail shop**

- 1. Name of retail shop \_\_\_\_\_ Respondent name \_\_\_\_\_ (Confidential)
- 2. Gender of the respondent    A. Male    B. Female

**4. Position of the respondent in the shop:** A. Owner B. Employee C. Relative **Other** \_\_\_\_\_

**5. Educational status of the respondent**

A. No formal education B. 1-8 C. 9-10 D. Preparatory school E. Diploma/Degree

**Section C: Reception, transportation, packaging condition and cottage cheese quality test**

1) From whom do you receive or buy cottage cheese?

A. Farm market B. Household producers C. Factories or processors D. Other, specify

2) How is cottage cheese transported from processor to retail point?

A. Trekking on foot B. Three-wheel drive "Bajaj" C. Animal drawn cart D. Refrigerated vehicles/ cold chain E. Own transport vehicle F. Public transport G. Other, specify

**3) Observational: What is the packaging material in which cottage cheese is received by Retailer from farm market or processor?**

A. Plastic bucket B. Plastic bottles C. Poly ethylene plastic container "festal" **D. Other, specify**

**4. Observational: What does the quality of packaging materials looks like?** A. Good B. Poor

**Section D: Storage conditions of cottage cheese**

• Do you keep cottage cheese in a refrigerator until sell? A. Yes B. No

• Do you have a separate refrigerator for cottage cheese (dairy product)? A. Yes B. No

3. What is the temperature of the refrigerator? \_\_\_\_\_ check using thermometer r in °C

4. Do you have backup generator to be used in case when electric power is out? A. Yes B. No

**Section E: Additional comments from the respondent about milk retail job**

Any comment the respondent gives here about what kind of support the retail shop needs or any other things related to cottage cheese retail job

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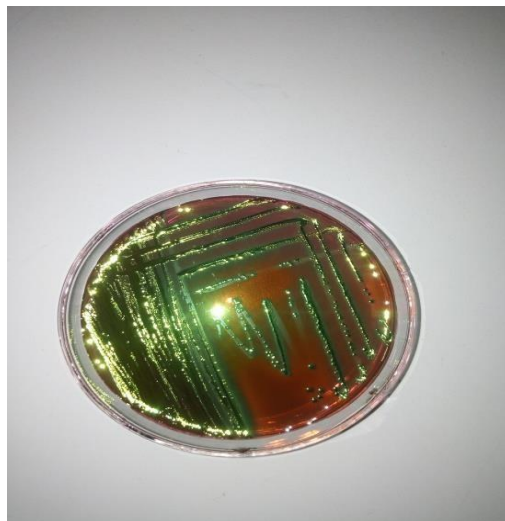
*Thank you for your time!!!*

**Annex 8: Pictures of coliform and *E. coli* on petrifilm**

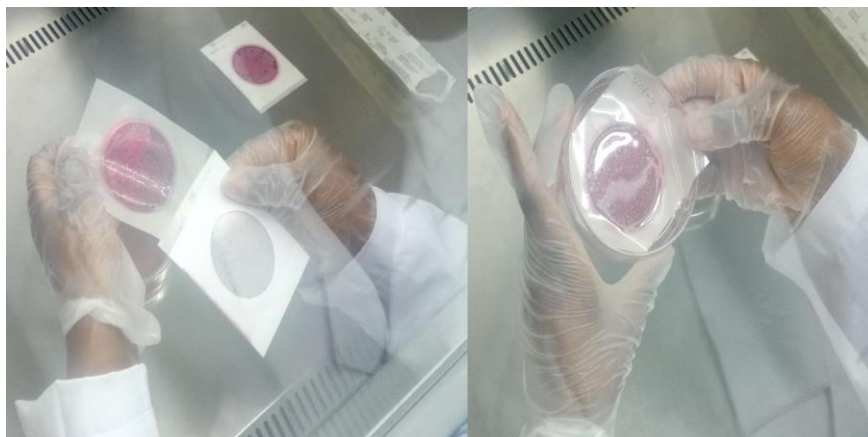


Total coliforms (Red colonies with gas) and *E. coli* (blue colonies with gas)

**Annex 9: Pictures for the isolation of STEC and O157:H7**



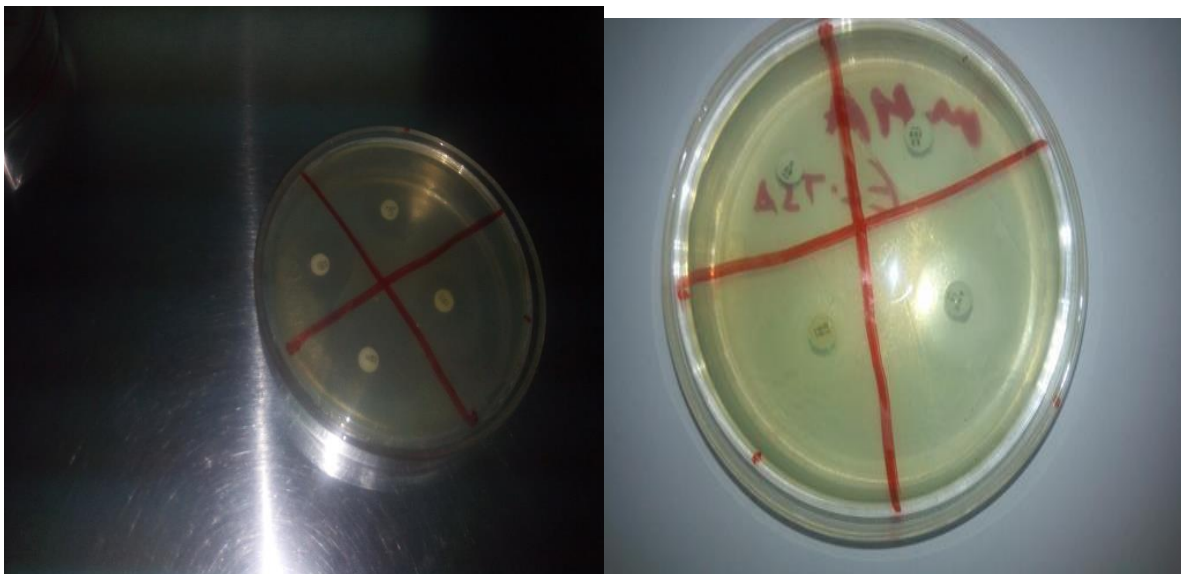
*E. coli* on Eosin Methylene Blue agar (EMB)





Characteristics of pathogenic *Escherichia coli* on Sorbitol MacConkey Agar supplemented with cefixime and potassium tellurite (CT-SMAC). Note the whitish colonial appearance (non-sorbitol fermenters) of *E. coli* O157:H7

**Annex10:** Pictures for the Antimicrobial test



### **Annex 11: Standard Operating Procedure for Enumeration of Aerobic plate counts**

#### **Method reference**

U.S. Food & Drug Administration Center for Food Safety & Applied Nutrition;  
Bacteriological Analytical Manual, 2001

#### **Method principle**

The aerobic colony count estimates the number of viable aerobic bacteria per gm or ml of products. A portion of the diluted sample mixed with a specified agar medium and incubated under specific temperature for 48 hr. It is assumed that each viable aerobic bacterium will multiply under these conditions and give rise to colonies.

### **Equipment and materials**

1. Work area, level table with ample surface in room that is clean, well-lighted (100 foot-candles at working surface) and well-ventilated, and reasonably free of dust and drafts. The microbial density of air in working area, measured in fallout pour plates taken during plating, should not exceed 15 colonies/plate during 15 min exposure.
2. Storage space, free of dust and insects and adequate for protection of equipment and supplies
3. Petri dishes, glass or plastic (at least 15 x 90 mm)
4. Pipets with pipet aids (no mouth pipetting) or pipettors, 1, 5, and 10 ml, graduated in 0.1 ml units
5. Dilution bottles, 6 oz (160 ml), borosilicate-resistant glass, with rubber stoppers or plastic screw caps
6. Pipet and petri dish containers, adequate for protection
7. Circulating water bath, for tempering agar, thermostatically controlled to  $45 \pm 1^{\circ}\text{C}$
8. Incubator,  $35 \pm 1^{\circ}\text{C}$ ; milk,  $32 \pm 1^{\circ}\text{C}$
9. Colony counter, dark-field, Quebec, or equivalent, with suitable light source and grid plate
10. Tally register
11. Dilution blanks,  $90 \pm 1$  ml Butterfield's phosphate-buffered dilution water; milk,  $99 \pm 2$  ml
12. Plate count agar (standard methods)
13. Refrigerator, to cool and maintain samples at  $0-5^{\circ}\text{C}$ ; milk,  $0-4.4^{\circ}\text{C}$
14. Freezer, to maintain frozen samples from  $-15$  to  $-20^{\circ}\text{C}$
15. Thermometers (mercury) appropriate range; accuracy checked with a thermometer certified by the National Institute of Standards

### **Procedure:**

#### **Sample preparation**

- Weigh 10g or 10ml of the sample into a sterile 250ml Erlenmeyer flask; marked to indicate 100ml volume.
- Add sterile normal saline to 100ml mark.
- Dissolve and shake thoroughly with 90ml of normal saline or appropriate diluents.

**Inoculation:**

- Pipette 1ml of the food homogenate of each dilution into each of the appropriately marked duplicate dishes.
- Pour 15-20ml of the molten PCA kept at  $45 \pm 1$  °C of water bath into each duplicate Petri dishes.

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

**Dilution factor:**  $1 \times 10^1$ ,  $1 \times 10^2$ ,  $1 \times 10^3$  etc

- Mix homogenate by shaking then pipette 1ml into a tube (labeled  $10^{-2}$ ) containing 9ml of normal saline.
- Mix carefully by aspirating 10 times with a pipette.
- From the  $10^{-2}$  dilution, transfer with the same pipette 1ml to the tube (labeled  $10^{-3}$ ) containing 9ml of the diluents, Mix with a fresh pipette
- Repeat the above procedure until the required numbers of dilutions are made.

**Incubation**

- Incubate the dishes, inverted, at  $35 \pm 1$ °C or for dairy products at  $32 \pm 1$ °C for  $48 \pm 2$  hr.

**NB:** Avoid excessive humidity in the incubator to reduce the tendency for spreader formation, but prevent excessive drying of the medium by controlling ventilation and air circulation. Agar in plates should not lose weight by more than 15% during  $48 \pm 2$  hours of incubation.

**Computing and recording counts**

To avoid creating a fictitious impression of precision and accuracy when computing APC, report only the first two significant digits. Round off to two significant figures only at the time of conversion to SPC. For milk samples, when plates for all dilutions have no colonies, report APC as less than 25 colonies estimated count. Round by raising the second digit to the next highest number when the third digit is 6, 7, 8, or 9 and use zeros for each successive digit toward the right from the second digit. Round down when the third digit is 1, 2, 3, or 4. When the third digit is 5, round up when the second digit is odd and round down when the second digit is even.

**Example:**

Calculated count	APC
12,700	13,000
12,400	12,000
15,500	16,000
14,500	14,000

**1. Plates with 25-250**

$$N = \frac{\sum c}{[n_1 + n_2(0.1)]d}$$

a. Calculate the APC as follows

$$N = \frac{\sum c}{[n_1 + n_2(0.1)]d}$$

- Where:** C = is the sum of colonies on all plates counted  
V = is the volume applied to each plate  
n<sub>1</sub> = is the number of plates counted at first dilution.  
n<sub>2</sub> = 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.

$$31 + 31 \text{ colonies} / 0.0015 \text{ ml} = 4.1 \times 10^4$$

b. When counts of duplicate plates fall within and without the 25-250 colony range, use only those counts that fall within this range.

**2. All plates with fewer than 25 CFU.** When plates from both dilutions yield fewer than 25 CFU each, record actual plate count but record the count as less than  $25 \times 1/d$  when d is the dilution factor for the dilution from which the first counts were obtained

**Example**

Colonies		
1:100	1:1000	EAPC/ml (g)
18	2	<2,500
0	0	<2,500

**3. All plates with more than 250 CFU.** When plates from both 2 dilutions yield more than 250 CFU each (but fewer than 100/cm<sup>2</sup>), estimate the aerobic counts from the plates (EAPC) nearest 250 and multiply by the dilution.

**Example**

Colonies
----------

1:100	1:1000	EAPC/ml (g)
TNTC	640	640,000

*TNTC*, too numerous to count.

*EAPC*, estimated aerobic plate count.

4. *All plates with spreaders and/or laboratory accident.* Report respectively as Spreader (SPR), or Laboratory Accident (LA).
5. *All plates with more than an average of 100 CFU per sq cm.* Estimate the APC as greater than 100 times the highest dilution plated, times the area of the plate. The examples below have an average count of 110 per sq cm.

**Example**

Colonies/Dilution		
1:100	1:1000	EAPC/ml (g)
TNTC	7,150 <sup>(a)</sup>	>6,500,000 EAPC <sup>(b)</sup>
TNTC	6,490	>5,900,000 EAPC

<sup>a</sup> Based on plate area of 65 cm<sup>2</sup>

<sup>b</sup> EAPC, estimated APC

<sup>c</sup> Based on plate area of 59 cm<sup>2</sup>

**Verification:** If there is 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:** Express the result in cfu per g or ml (if a liquid sample)

**Method of calculation: general case (counting of total colonies or typical colonies)**

For a result to be valid, it is generally considered necessary to count the colonies on at least one dish containing at least 10 colonies (total colonies, typical colonies or colonies complying with identification criteria (ES ISO 7218, 2015)

Calculate the number *N* of microorganisms present in the test sample as a weighted mean from two successive dilutions using Equation (1):

$$N = \frac{\sum c}{V \times 11 \times d}$$

Where:

$\sum C$  is the sum of the colonies counted on the two dishes retained from two successive dilutions, at least one of which contains a minimum of 10 colonies

*V* is the volume of inoculum placed in each dish, in milliliters;

$d$  is the dilution corresponding to the first dilution retained [ $d = 1$  when the undiluted liquid product (test sample) is retained (ES ISO 7218, 2015)]

Round off the calculated result to two significant figures. When doing this, if the third figure is less than 5 do not modify the preceding figure; if the third figure is greater than or equal to 5, increase the preceding figure by one unit.

Express the result preferably as a number between 1, 0 and 9, 9 multiplied by the appropriate power of 10, or a whole number with two significant figures.

Report the result as the number  $N$  of microorganisms per millilitre (liquid products) or per gram (other products).

EXAMPLE Counting has produced the following results:

- at the first dilution retained (10–2): 168 colonies;
- at the second dilution retained (10–3): 14 colonies.

$$N = \frac{\sum C}{V \times 1,1 \times d} = \frac{168 + 14}{1 \times 1,1 \times 10^{-2}} = \frac{182}{0,011} = 16\,545$$

Rounding off the result as specified above, the number of microorganisms is 17 000 or  $1,7 \times 10^4$  per milliliter or per gram of product.

## **Annex 12: Standard Operating Procedure for Enumeration of *E. coli*/coliforms**

### ***E. coli* and coliforms quantification using Petrifilm Methods**

**Introduction:** This Standard Operating Procedure (SOP) was created based upon FDA BAM (Hunt, Abeyta, and Tran, 2001), 3M™ Petrifilm™ Product Instructions, and 3M™ *E. coli*/coliform Petrifilm Interpretation guide (3M™ Food Safety), for the detection and enumeration of *Escherichia coli* and coliforms in milk and other dairy products. The *E. coli*/coliform Petrifilm Count Plate contains modified violet red bile (VRB) nutrients and a tetrazolium indicator to facilitate in counting, also contains a  $\beta$ -glucuronidase indicator for *E. coli*. The plate can yield results in 24-48 hours of incubation after inoculation. The following outlines the procedures for trained personnel working within approved laboratories associated with these project teams.

#### **Safety precautions**

*Escherichia coli* and coliforms are generally categorized as Biosafety Level 2 pathogens and Biosafety Level 1 non-pathogenic indicators, respectively. Nevertheless, CDC guidelines for handling Biosafety Level 1 pathogens should be followed whenever samples are tested for *E. coli* and coliforms. A Class II laminar flow biosafety cabinet is recommended for procedures in which infectious aerosols or splashes may be created. The Safety Data Sheet (SDS) must be obtained from the manufacturer for the media, chemicals, reagents and microorganisms used in the analysis. The personnel who will handle the material should read the SDS prior to startup.

\*\*\*DO NOT use this plate for the detection of *E. coli* O157. Because most *E. coli* O157 strains are atypical, for example they are glucuronidase negative, they will not produce a blue color, and will not be detected on *E. coli*/coliform Petrifilm. \*\*\*

### **Responsibilities**

- The study coordinator is responsible for ensuring all equipment required to follow this SOP is available, safe, and in good working order.
- The field coordinator is responsible for ensuring all relevant meta-data on the samples is recorded when collected, the delivery of samples and sample information to the laboratory, and temporary storage of samples in the project freezer or fridge as appropriate.
- Each study laboratory technician and student is responsible for documenting receipt of samples and ensuring that they are appropriately stored before samples are tested.
- Each study laboratory technician and student is responsible for documenting appropriate information in regards to testing for each sample and recording of test results in the format decided on by the project.

### **Equipment and materials**

- Autoclave or sterilization equipment
- Biosafety tip autoclave bags
- Biosafety autoclave bags
- Autoclave tape
- *E. coli*/coliform Petrifilm Count Plate
- Incubators capable of operating at  $35 \pm 1$  °C
- Pipettor 100-1000  $\mu$ l
- Sterile pipette tips 1000  $\mu$ l
- Sterile serological pipettes 10 ml
- Serological pipette controller or silicone pipette filler
- PBS or 0.85% saline
- Glass or conical tubes 15 ml
- Refrigerator ( $8 \pm 3$  °C)
- Sample labeling tape or marker

### **Procedure:**

Below-described is a procedure for analysis of dairy samples with expected high level of background microbial contamination.

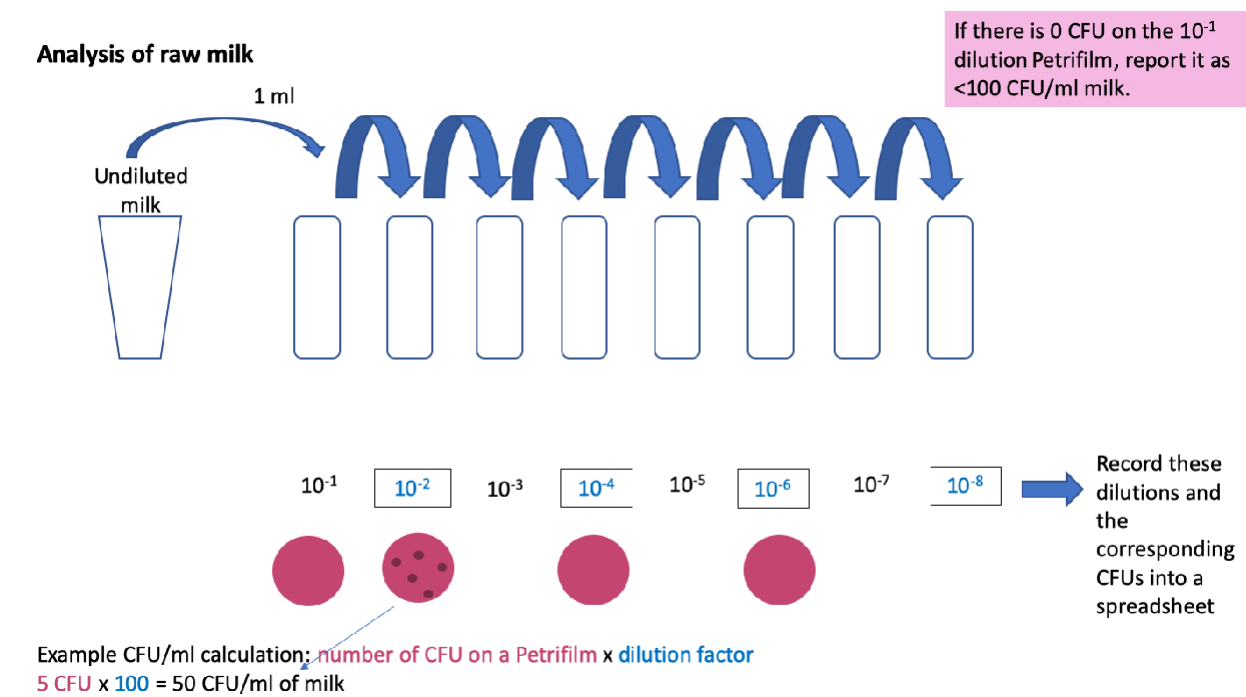
Ensure that the pH of the saline is between 6.6 – 7.2.

The below-outlined dilution and plating schemes are recommended based on preliminary tests of Ethiopian raw and pasteurized milk, and cottage cheese by the ENSURE project.

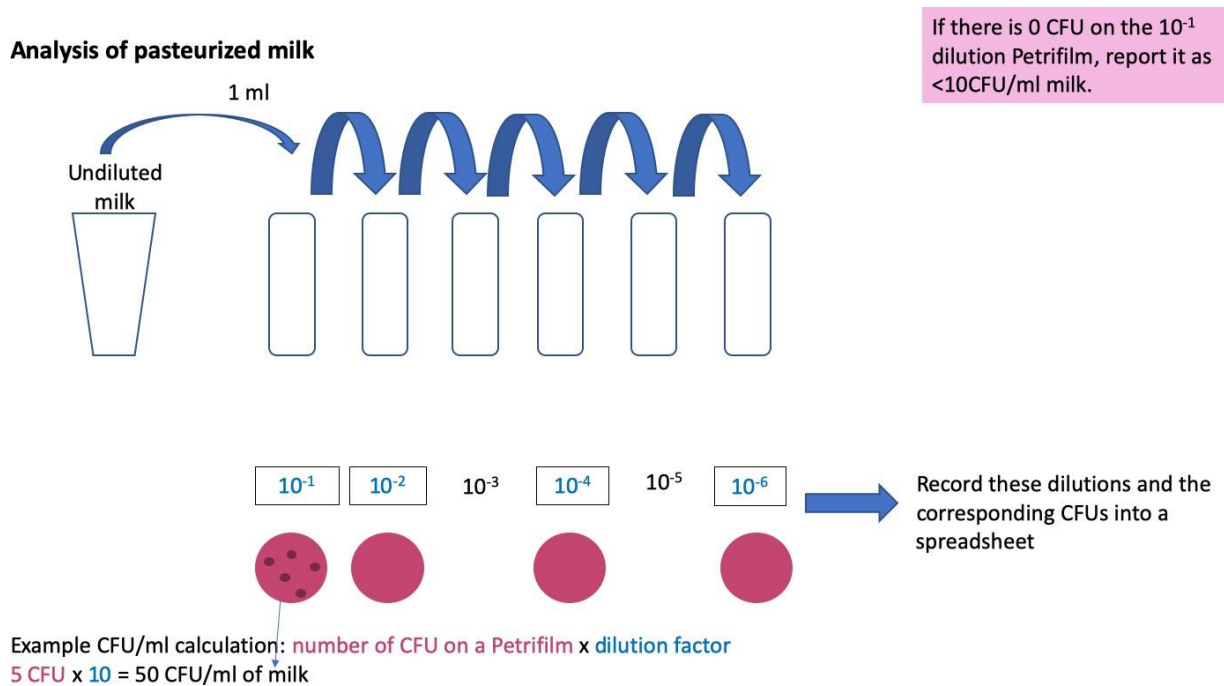
### **Milk samples**

**Sample preparation:** Liquid samples (such as milk) can be readily used for serial dilutions. Pipette up and down or gently vortex the sample to ensure that the sample is homogenized.

1. Prepare serial dilutions by transferring 1 ml of the sample to a sterile tube with 9 ml of sterile PBS or 0.85% saline. Vortex to homogenize.
2. Transfer 1 ml from the tube into the next tube and repeat the process 7 more times for raw milk samples (see Fig. 1) and 5 more times for pasteurized milk samples (Fig. 2).
3. Plate 1 ml of dilutions  $10^{-2}$ ,  $10^{-4}$ ,  $10^{-6}$ , and  $10^{-8}$  for raw milk (Fig. 1) and  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-4}$ ,  $10^{-6}$  for pasteurized milk samples (see Fig. 2) onto pre-labeled 3M™ *E.coli*/ coliform Petrifilm™, in duplicates.
  - Lift the top film, hold the pipette perpendicular to the inoculation area and dispense 1 ml of sample suspension.
  - To prevent trapping air bubbles, carefully roll the top film down.
  - Optional: Place the Petrifilm spreader with the flat side down on the center of the plate. Press gently on the center of the petrifilm spreader to distribute the sample evenly. Spread the inoculum over the entire growth area before the gel is formed. Do not slide or twist the petrifilm spreader across the film, as this will result in sample overflow.
  - Remove the spreader and leave the *E. coli*/coliform Petrifilm undisturbed for at least one minute to allow for the gel to form.



**Fig. 1:** Dilution and plating scheme for raw milk samples.



**Fig. 2:** Dilution and plating scheme for raw milk samples.

### Cottage cheese samples

Sample preparation: Homogenize 10 g of cottage cheese in 90 ml of PBS or 0.85% saline. This homogenate represents 1:10 dilution (see Fig. 3).

1. Prepare serial dilutions by transferring 1 ml of the homogenate to a sterile tube with 9 ml of sterile PBS or 0.85% saline. Vortex to homogenize.
2. Transfer 1 ml from the tube into the next tube and repeat the process 5 more times (see Fig. 3)
3. Plate 1 ml of dilutions  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-4}$ ,  $10^{-6}$  (see Fig. 3) onto pre-labeled 3M™ E.coli/coliform Petrifilm™, in duplicates.
  - Lift the top film, hold the pipette perpendicular to the inoculation area and dispense 1 ml of sample suspension.
  - To prevent trapping air bubbles, carefully roll the top film down.
  - Optional: Place the Petrifilm spreader with the flat side down on the center of the plate. Press gently on the center of the petrifilm spreader to distribute the sample evenly. Spread the inoculum over the entire growth area before the gel is formed. Do not slide or twist the petrifilm spreader across the film, as this will result in sample overflow.
  - Remove the spreader and leave the *E. coli*/coliform Petrifilm undisturbed for at least one minute to allow for the gel to form.

### Analysis of cheese

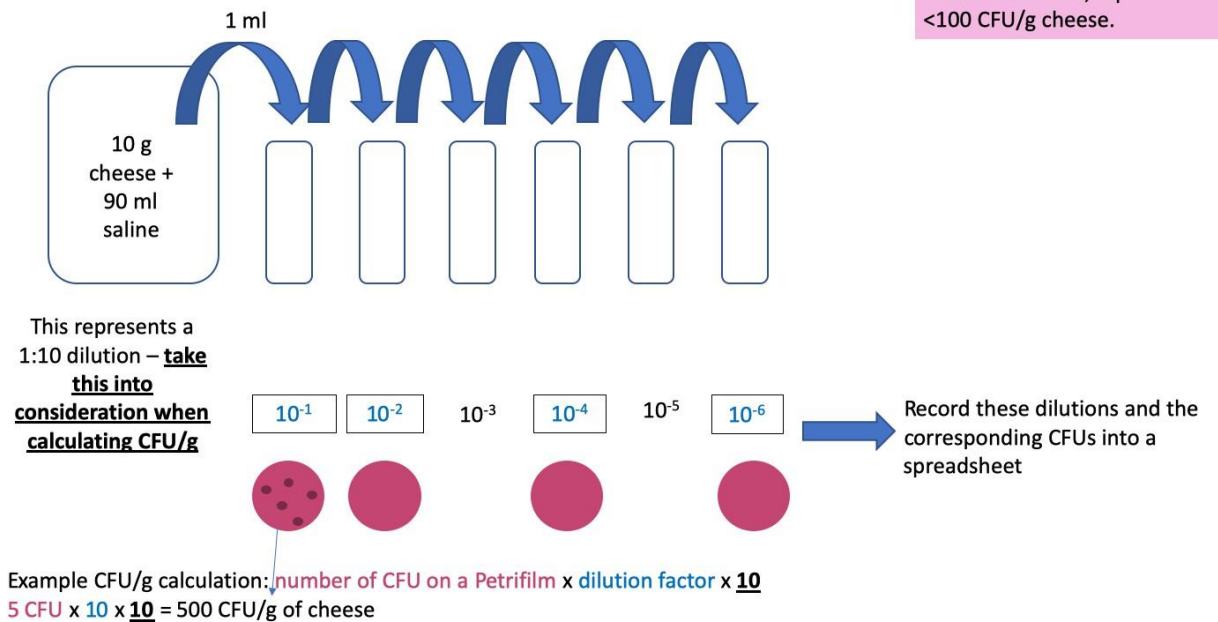


Fig. 3: Dilution and plating scheme for cottage cheese samples.

**\*Refer to the 3M Petrifilm Interpretation Guide for a diagram of the procedures (3M Food Safety, 2017).**

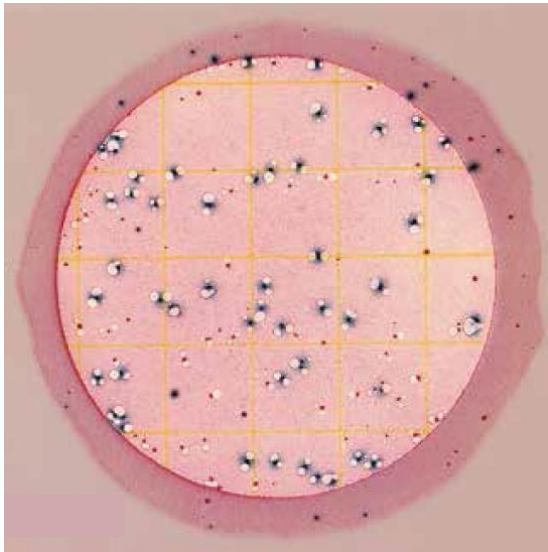
### **Incubation:**

Incubate the petrifilm plates in a horizontal position with the clear side up in stacks of no more than 20 plates at  $35^{\circ}\text{C} \pm 1^{\circ}\text{C}$  for 24 hours  $\pm$  2 hours. Count coliform colonies after 24-hour incubation (based on a recommendation of a 3M technical specialist). Incubate for additional 24 h  $\pm$  2 hours (48 hours  $\pm$  4 hours total) at  $35^{\circ}\text{C} \pm 1^{\circ}\text{C}$  and count *E. coli* colonies (*E. coli* colonies may need up to 48 h to grow large enough to detect blue color of colonies).

### **Interpretation:**

**Repeat the following steps for all dilutions plated.**

1. The interpretation of colonies on the *E. coli*/coliform Petrifilm according to the AOAC Official Methods (998.08 and 991.14) is as follows:  
Blue or purple colonies associated with entrapped gas- confirmed *E. coli* colony (See Figure 2).  
Red colonies associated with entrapped gas- conformed coliform colony (See Figure 2).  
Colony with no associated entrapped gas are not counted as *E. coli* or coliforms.  
The total coliform count is the number of both red and blue colonies associated with gas at 24 hours. Refer to Figure 3 for examples of associated entrapped gas.

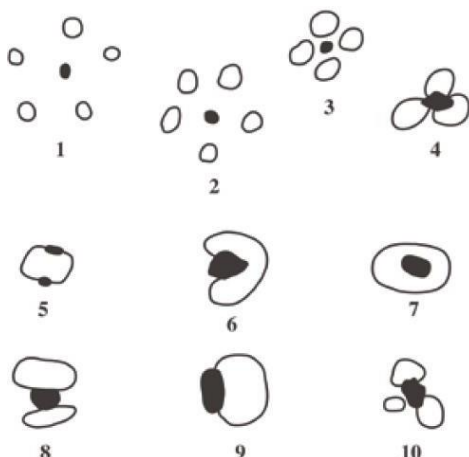


**Figure 2:** *E. coli* count is 49 (blue colonies with gas bubbles) and total coliform count is 87 (red AND blue colonies with gas bubbles).

Taken from 3M™ Petrifilm *E. coli*/Coliform Count Plate Interpretation Guide.

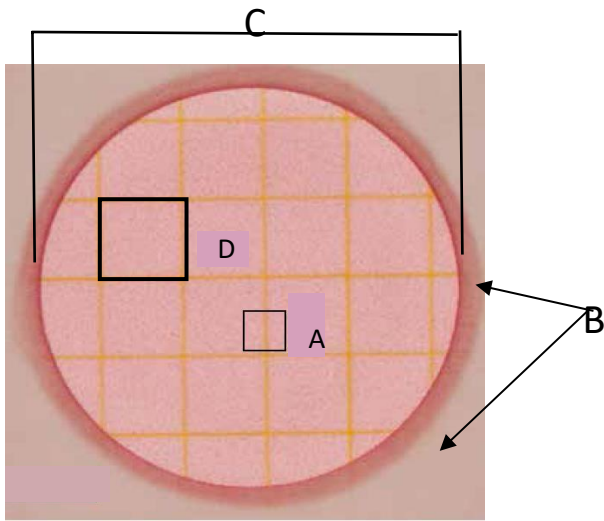
**Do not count colonies on the foam dam (See Figure 4) as they are outside the selective influence of the media in the growth area. Take care to not count artifact bubbles (not associated with a colony).**

2. The circular growth area is approximately 20 cm<sup>2</sup>. If there are more than 150 colonies, you can estimate the number of colonies by counting the number of colonies in one or more representative squares and calculating the average number of colonies. Multiply the average number by 20 to determine the estimated count per plate.
3. High bacterial load can result in a deepening of the gel color and several, small indistinct colonies and many gas bubbles. A high concentration of coliforms can turn the gel a dark red color. A high concentration of *E. coli* can turn the gel a dark blue color. If this occurs, record results as “too numerous to count” (TNTC) and look at or prepare additional plates with a higher dilution. A high concentration of non-coliform organisms can turn the gel yellow.
4. If colonies are need for further testing and identification, lift the film cover and pick a colony from the gel.
5. If the plates cannot be counted within 1 hour of removal from the incubator, they may be stored for later by freezing in a sealable container at temperatures  $\leq -15^{\circ}\text{C}$  for no longer than one week.



**Figure 3:** Examples of entrapped gas bubbles associated with colonies. All should be included in enumeration.

Taken from 3M™ Petrifilm *E. coli*/Coliform Count Plate Interpretation Guide.



**Figure 4:** Examples of a *E. coli*/coliform Petrifilm with no colonies. Artifact bubble (A), foam dam (B), growth area (C), and square (D) are labeled,

**\*Refer to the 3M™ Petrifilm *E. coli* / Coliform Count Plate Interpretation Guide for additional help.**

**3M™ Petrifilm EC Plate Storage and Maintenance:**

Store unopened *E. coli*/coliform Petrifilm pouches in a refrigerator or frozen at temperatures  $\leq 8^{\circ}\text{C}$ . Just prior to use, allow for the unopened pouches to reach room temperature before opening. Return unused *E. coli*/coliform Petrifilm to pouch. Seal the pouch by folding the end of the pouch over and using adhesive tape. **To prevent exposure to moisture, do not refrigerate opened pouches,** instead store resealed pouches in a cool dry place for no longer than four weeks.

**Annex 13: DNA extraction, PCR, gel electrophoresis and gel imaging**



**Anenx 14.** Ethical approval and clearance for this study was obtained from Addis Ababa University (AAU)

COLLEGE OF NATURAL & COMPUTATIONAL SCIENCES  
Addis Ababa University



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Ref. No.  
ቁጥር: CNSDO/296/12/2020  
Date:  
ቀን: January 23, 2020

**To Whom It May Concern**

The College of Natural & Computational Sciences Institutional Review Board (CNS-IRB) Committee in its meeting held on 20/12/2019 Minute No. IRB/42/2019 has examined the project proposal entitled “**Assessment of Risk Factors for Microbial and Chemical Contamination of Milk and Cottage Cheese Across the Dairy Value in Ethiopia**” by Dr. Ashagrie Zewdu, from the Addis Ababa University.

The proposal is approved for implementation.

With regards,

Addisalem Abathun, PhD  
Dean, College of Natural & Computational Sciences



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Please Quote our reference number in you correspondence