

UNIVERSITY OF GHANA - LEGON



**PREVALENCE OF ESCHERICHIA COLI O157:H7, LISTERIA MONOCYTOGENES
AND STAPHYLOCOCCUS AUREUS IN READY-TO-EAT FRESH MILK AND FRESH
MILK PRODUCTS IN MILK MARKETS IN ACCRA.**

BY

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(MPHIL) IN FOOD SCIENCE**

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DECLARATION

I Paa Kweku Baffoe, declare that this work was done by me under supervision in the Department of the Nutrition and Food Science, University of Ghana, Legon.

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DEDICATION

This dissertation is dedicated to my parents, Victor and Dorcas Atta-Baffoe, for their support, encouragement and prayers throughout my entire life.



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ABSTRACT

The fresh milk industry in Ghana is a source of livelihood for a significant proportion of small-scale farming households in the country. However, lack of consumer food safety assurance of fresh milk and fresh milk products have limited business growth of this sector. Reports on lack of cooling infrastructure, unhygienic handling, inadequate processing and post-process contamination contributes to microbial spoilage in dairy products leading to loss of large volumes of milk in developing countries.

This study therefore sought to assess the microbiological quality and hygiene of raw milk from assemblers and fresh milk products from five informal milk markets. This was accomplished by subjecting randomly sampled fresh milk and fresh milk products (22 *brukina*, 18 boiled milk, 15 raw milk, 8 *nunu*, 24 yoghurt, 17 raw *wagashi* and 13 fried *wagashi*) to standard cultural methods of microbial assessments. Products were tested for concentration of index and hygiene indicators including total coliforms, fecal coliforms, *Escherichia coli* and Staphylococci. Confirmation of suspected pathogens was done with biochemical tests, API 20E and Matrix-Assisted Laser Desorption/Ionization Time Of Flight (MADLI-TOF) mass spectrometry. Quantitative Polymerase Chain Reaction (qPCR) was done to confirm the prevalence of *E. coli* O157:H7, *Listeria monocytogenes* and *Staphylococcus aureus* in the ready-to-eat (RTE) dairy products tested. Mean counts were reported in Log cfu/g or Log cfu/ml using Microsoft Excel (2016). Significant differences between total coliforms, fecal coliforms, *E. coli* and Staphylococci counts was determined using Analysis of Variance (ANOVA) with a P value of <0.05 using IBM SPSS version 21.

The study in general identified insufficient heat treatment used to pasteurize the dairy products, poor hygienic practices and possible post-process contamination as reasons for the poor

microbiological quality of the samples tested. The quality of the raw milk had a direct effect on the quality of the final milk products. There was statistical similarity ($P \leq 0.05$) in the coliform content between raw milk (3.64 ± 2.56 Log cfu/ml) and boiled milk (3.61 ± 3.06 Log cfu/ml) which signified insufficient heat treatment used in processing the boiled milk. The study identified a general high prevalence of coliform contamination (67%), with raw wagashi having the highest coliform and *Escherichia coli* concentrations of 5.90 ± 3.79 Log cfu/g and 2.61 ± 2.65 Log cfu/g respectively. Fried wagashi emerged as the only product which was safe from coliform contamination. The staphylococci concentration of the products analyzed were within the acceptable (3.0 Log cfu/ml/g) limits. *L. monocytogenes* was not detected in any of the products analyzed using cultural methods. Results from qPCR indicated high prevalence of *E. coli* O157:H7 in boiled milk (66%), *nunu* (37%) and *brukina* (14%). Boiled milk had a 42.8% *S. aureus* prevalence and 11.11% prevalence of *L. monocytogenes*. The results from the viability tests suggests shortfalls in the processing of the RTE fresh milk products in the local milk markets. The presence of significant pathogens in the product has implications on food security with respect to food safety.

Overall microbiological analysis conducted suggests a poor quality of RTE fresh milk products sold in the informal markets in Accra. This indicates an urgent need for intervention measures with highlights on effective pasteurization of raw milk and training of the various stakeholders in the dairy value chain on hygienic handling to prevent post-process contamination.

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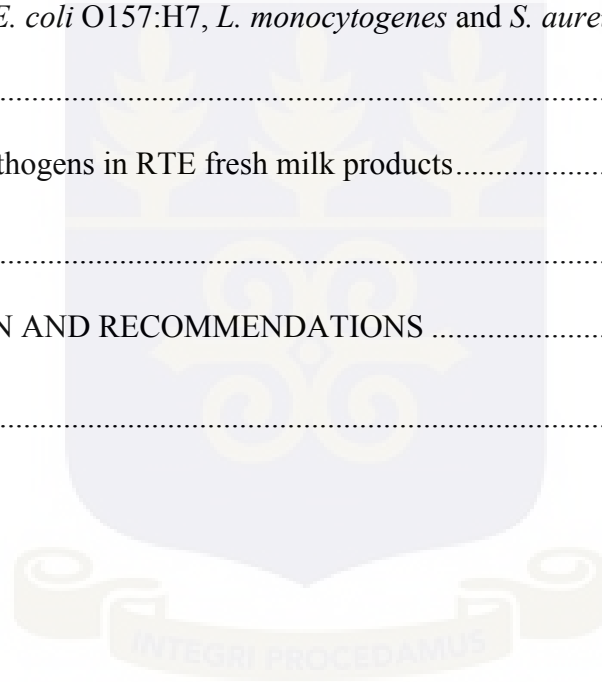


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CHAPTER ONE

1.0 INTRODUCTION

Food Safety has emerged as an important global issue with highlights on trade and public health implications (Haghi *et al*, 2015). Consumers worldwide are becoming more aware of the safety of the food products they consume. Raw milk marketed by small-scale farmers for the production of various dairy products is often of poor quality due to initial bacterial load (Nada *et al*, 2012). Bacterial pathogens have been reported to be major agents of milk borne diseases, posing a major health problem in developing countries (Haghi *et al.*, 2015). Although food safety technologies have improved over the years, reports of foodborne illnesses and foodborne outbreaks due to the presence of foodborne pathogens have increased (Fusco & Quero, 2014). Developing countries face a greater risk with issues including inefficient processing operations and improper storage of dairy products which serves as a gateway for pathogen transmission (Dhanashekar *et al.*, 2012). Amongst the milk borne pathogens commonly associated with high risk ready-to-eat (RTE) foods, *Staphylococcus aureus*, *Escherichia coli* O157:H7 and *Listeria monocytogenes* are significant (Claeys *et al*, 2013). The presence of these organisms is often indicative of poor personal hygiene, insufficient processing and post-process contamination (Millogo *et al.*, 2010). They pose major threats to the health of consumers, with risks ranging from gastroenteritis to life-threatening conditions including hemolytic uremic syndrome, spontaneous abortion and even death (Altekruse *et al*, 1997). Several studies have reported the presence of such pathogens in informally traded RTE foods in developing countries (Appiah, 2012; Ayebo 1973; Donkor *et al*, 2007; Glover & Akabanda, 2010; Gran, 2003). The informal milk sector in developing countries escapes food safety regulation, therefore, food processing, retail and handling malpractices go unchecked (Belli *et al.*, 2013). The fresh milk industry in Ghana is predominantly informal, small-scale, resource

deficient and unregulated (Omore *et al*, 2004). The unhygienic sourcing, processing, handling and retail activities typical of the fresh milk value chains in Ghana (Otchere & Okantah, 2001), increases risk of contamination with foodborne pathogens, leading to low patronage of fresh milk (Omore *et al*, 2004). Considering the vulnerability of milk as a perfect growth medium for several micro-organisms, characteristics of fresh milk stakeholders such as low or no formal education, poor sanitation facilities (Gidiglo, 2014), their prevailing practices including not washing the teats of the animals before milking and milking animals receiving acaricide, a pesticide, treatment (Addo *et al.*, 2011) contributes to the poor microbiological quality of informally traded fresh milk products. It is therefore important to investigate the prevalence of key milk safety pathogens in locally sourced milk. Information concerning microbial risk factors and hazards associated with raw milk consumption together with its risk profile in informally traded milk helps in the development of appropriate the quality assurance systems and microbiological criteria for the local dairy industry (Haghi *et al.*, 2015).

1.1 Study Rationale

Studies have pointed to the poor microbiological quality of locally sourced milk and milk products in Ghana. There is still however, a paucity of evidence to reliably assess the safety of locally sourced milk and milk products available at the point of sale in the informal milk markets in Ghana. Considering WHO efforts to increase domestic milk production and consumption in Africa (Ghana News Agency, 2003), it is important also to be aware of the food safety risks associated with locally produced milk. Information from this study will be critical in assessing the microbiological safety of informally retailed fresh milk products, and proposing interventions aimed at improving microbiological safety of fresh milk products marketed informally.

1.2 Main Objective

The study was designed to investigate the prevalence of *Staphylococcus aureus*, *Escherichia coli* O157:H7 and *Listeria monocytogenes*, as indicators of the safety of locally sourced fresh milk products from informal markets in Accra.

1.3 Specific Objectives

The specific objectives of the study were to:

- I. Assess the hygienic quality of the RTE fresh milk products marketed in Accra using culture based methods.
- II. Determine the prevalence of *Staphylococcus aureus*, *Escherichia coli* O157:H7 and *Listeria monocytogenes*, in selected RTE fresh milk products from informal markets using molecular methods.



CHAPTER TWO

2.0 Literature Review

2.1 Dairy Industry in Ghana

Milk in Ghana is predominantly sourced from cattle which are usually owned by Ghanaians but herded by men belonging to the nomadic Fulani ethnic group (Okantah *et al.*, 1997). The animals are mainly kept for meat and the milk is usually given as payment to the Fulani for their services (Okantah *et al.*, 1997). A review published by Ministry Of Food And Agriculture, (MoFA, 2013) reported an estimate of 1.5million cattle in Ghana. The common breeds of cattle found in Ghana include N'dama, West African Shorthorn, Sokoto Gudali and the Sanga breeds. The dominating breed is the West African shorthorn which constitutes 65% of the total population of cattle in Ghana (Gidiglo, 2014). Over the past few decades, dairy production in Ghana has been considered a major economic activity. The Fulani herdsmen are chiefly responsible for the milking of the animals while their wives take care of the sale and further processing of the milk into indigenous RTE products (Gidiglo, 2014).

2.2 Milk production and consumption in Ghana

The Ministry of Food and Agriculture (2016) reported that in the year 2013, Ghana produced 39,000 metric tons of milk but at the same time imported an estimated 38,187.5 metric tons of milk that same year. The milk imports were estimated by the UN trade statistics to have cost Ghana USD80 million (Yeboah, 2016).

The country predominantly consumes readily available condensed or sweetened condensed milk (Omore *et al.*, 2001), of which is mostly imported or constituted locally using imported materials. Consumer preference of imported milk over locally produced fresh milk clearly points out an

evidence of the mistrust of consumers towards the local dairy industry. Within the 24year period spanning from 1990 to 2014, the total population of Ghana saw a 28.7% increase with a 34% increase in the GDP per capita, as well as a 79.8% increase in food imports to the country (United Nations, 2015).

2.3 Small-scale dairying

Up to 75% of the milk marketed in developing regions is sold as raw or unpasteurized through informal channels (Bertu *et al.*, 2010). Without meeting national food standards, consumers purchase unpasteurized milk sold through informal channels because of reasons including relatively low prices in informal channels and limited availability of pasteurized milk (Blackmore *et al.*, 2015). The dairy industry in many parts of Ghana is considered relatively small-scale, under developed and mostly marketed through informal channels (Omore *et al.*, 2004). The profit margins generated in small-scale dairying is highly dependent on the form in which the milk is marketed, accessibility of the markets and the prevailing prices which is reflective to the number of intermediaries involved in the value chain (O'Mahony & Peters, 1987). Nonetheless, the nature of the dairy business, even though on a small-scale provides enough for their subsistence way of farming and also provides an average of 17 jobs comprising of both direct and indirect jobs per every 100 liters of milk produced (Otchere & Okantah, 2001). The dairy industry therefore holds great economic value to the country by not just creating jobs but also in increasing revenue and ultimately reducing poverty. The profit margins generated in small-scale dairying is highly dependent on the form in which the milk is marketed, accessibility of the markets and the prevailing prices which is reflective of the number of intermediaries involved in the value chain (O'Mahony & Peters, 1987).

2.4 Dairy Value Chain

Value chain refers to the full range of activities required to bring a product or service from conception, through intermediary phase of design, production, delivery to final consumers and final disposal after use (Kaplinsky & Morris, 2000). The dairy industry in Ghana consists of both formal and informal sectors. The two of the oldest home dairy industries, Fan milk Ghana and Nestle, both regarded as formal dairy industries, constitute their products locally using imported raw materials. Marketing and distribution of products by these industries make their way onto the market via different route from the ones by the informal dairy industries.

The informal local dairy industry usually comprises of several intermediaries between the sale on the farm until it reaches the consumer (Otchere & Okantah, 2001), with each activity causing an increase in the value of the product (Food and Agriculture Organization, 2017). The local dairy value chain in Ghana primarily consists of the milk producer, assemblers, processors, retailers and consumers (Gidiglo, 2014). The producers in Ghana are predominantly the nomadic Fulani herdsmen, just as reported in Burkina Faso by Millogo *et al* (2008) and in Nigeria by Yahuza (2001). Otchere and Okantah (2001) reported that the herd size in the southern savannah was larger as compared to the northern part of Ghana. They indicated that the herdsmen in general were employed as caretakers of the animals for the owners who were mostly not residing within the farming environment. Their only form of remuneration for their services comes from the sale of fresh milk from the animals. According to Yahuza (2001), the Fulani herdsmen are constantly moving towards locations with readily availability of water and pasture for their animals. Their wives take responsibility in processing the fresh milk into various products including local cheese, sour milk and sour yoghurt to help the keeping quality of the product. Sale of these products are mostly within walking distance from Fulani settlements. The milk marketing channels in Ghana,

as described by Omore *et al.* (2004), is characterized mainly by farm gate sales and hawking the left-over milk from house to house if not sold by noon. With these stakeholders playing a role in the value chain of milk in Ghana, it is only right to consider the exposure and quality of the product through the succession. Fresh milk produced on the farm is sold by the herdsmen or their wives either to the indigenous processors who make various products including *brukina*, *nunu*, *fura*, *wagashi* and yoghurt or it is sold to a group of people known as assemblers who bulk the milk for retailers, and then finally deliver to the consumers (Gidiglo, 2014). This value chain is similar to those in Uganda (Wozemba & Rashid, 2008), Ethiopia (Yilma *et al.*, 2011) and Kenya (Staal *et al.*, 2008), with the exception of the involvement of dairy cooperative societies playing a key role in the dairy value chain in the mentioned counties.

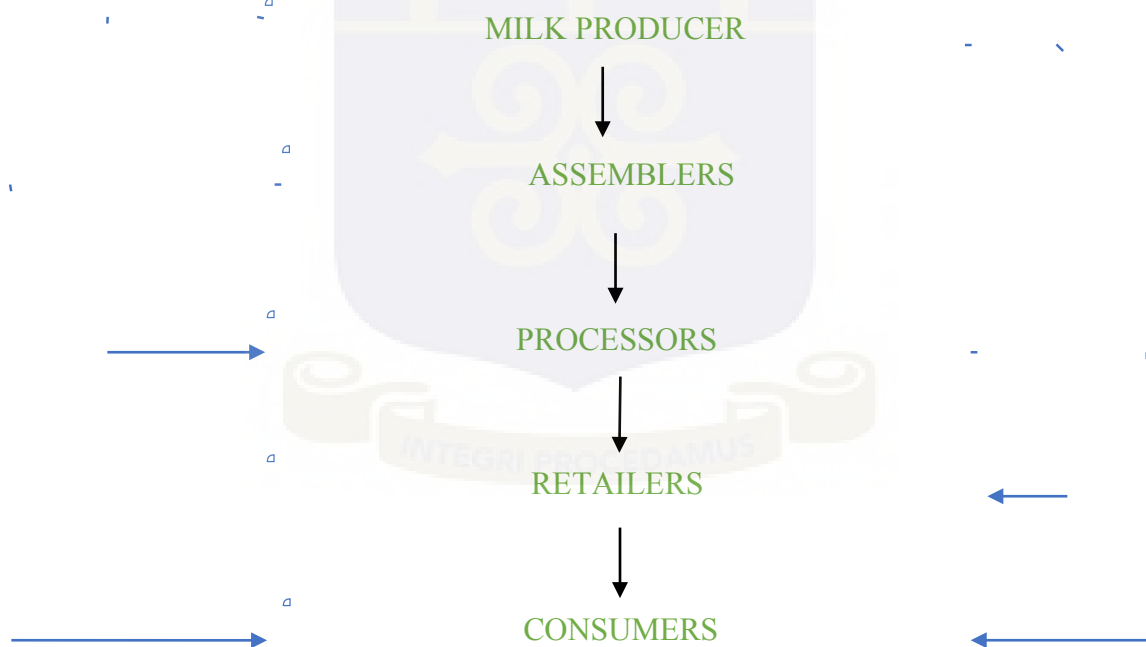


Figure 1: Flow scheme of dairy marketing chains in Accra plains

Source: (Gidiglo, 2014)

The milk value chain, unlike other agricultural commodity value chains in Ghana, presents equal opportunity gender wise, for the stakeholders involved (Lues *et al.*, 2010). A report by Omore *et al.* (2004) suggested a greater predominance of women along the informal milk value chain. Furthermore, a greater fraction of the women are either proprietors or family members of the proprietors. Omore *et al.* (2004) also submitted that only a low level of risk was associated with small-scale dairying in Ghana, contrary to what has been erroneously perceived in the past. The greatest risk in terms of poor quality was associated with the unmonitored large-scale dairy market agents, and the large numbers of intermediaries along the value chain. This, they explained, increased the number of handlers which inevitably increased exposure and contamination.

2.5 Milk Processing in Ghana

Processing of milk does not only add value to the fresh milk but also increases the usable life of the milk and reduces the risk of food borne illness through techniques such as pasteurization and fermentation. In many rural areas in the sub-Saharan regions in Africa, the milk produced is consumed at home as fresh or sour whilst the vendors in the urban markets process the surplus into dairy products including local cheese and butter. Without any formal education, the processors engage in traditional methods of milk processing which generally gives lower yields of final product, lower stability and hygienically unsafe products as compared to similar products produced in well-established dairy plants (O'Mahony & Peters, 1987). In Ghana, fresh milk produced is either consumed solely after heat treatment or processed into readily available foods including *brukina*, spontaneously fermented milk, *nunu* and indigenous cottage cheese also known as *wagashi*.

2.5.1 *Brukina*

Brukina is a complete meal consisting of a cereal base which is millet and has a characteristic sour taste originating from spontaneously fermented milk. It is considered an enriched meal and enjoyed by consumers of all age groups. The fermented milk is known for its probiotic effect (Tripathi & Giri, 2014) whilst the millet contributes calcium, manganese, protein, fats, carbohydrates, phosphorus, dietary fiber, antioxidants and B vitamins to the meal (Obilana, 1994). The milk base is often supplemented with powdered milk to help reduce the cost of production. It is often bottled and served chilled, or given in specific quantities depending on the request of the consumer, in which case, it is served in bowls or retail plastic bags by the retailers (Tawiah, 2015). Even though there are individual process variations in the processing of *brukina* according to processor's preference, both the millet and milk used in the production process undergo pre-treatments separately prior to production.

The process involved in the production of *brukina* is not regulated. Volumes of the added raw materials are based on the processors discretion and experience in the business. Sanitation and hygienic conditions around processing sites and utensils used are not checked and this poses a significant risk to the consumer. Figure 2 summarizes the traditional processing steps for *brukina* beverage.



Figure 2: Schematic flow of traditional *brukina* processing

Source (Tawiah, 2015)

2.5.2 *Nunu*

Naturally fermented milk, traditionally known as *nunu*, is a product made from unpasteurized cow milk, which has been allowed to spontaneously ferment overnight in vessels such as gourds, woven straw, animal skin, clay pots and calabash (Kurwijila, 2006). In Ghana, *nunu* is often sold in informal milk markets and amongst communities with natives from the three northern regions, where it is consumed as a staple food. It is typically served alone in clear plastic bags or with *fura* (a spontaneously fermented millet dough dumpling with spices, moulded balls and cooked), or roasted groundnuts or agglomerated millet in milk bars in urban, peri-urban and rural areas where it is consumed. The production makes use of raw milk only, or parts of a previous batch, indicating back slopping (Akabanda *et al.*, 2010). The process involved in the production of *nunu* is similar to *nono*, a traditionally processed fermented milk product in Nigeria (Uzeh *et al.*, 2006). The final product has a characteristic low viscosity and an off-white color due to the removal of fats from the final product. Figure 3 is the depiction of the process of making *Nunu*.

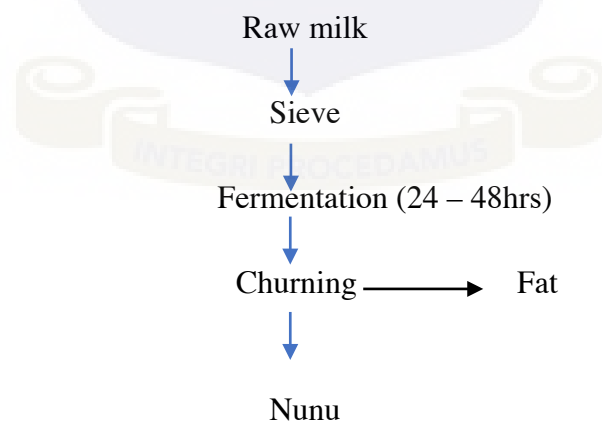


Figure 3: Schematic flow of traditional *nunu* processing

Source (Akabanda *et al.*, 2010)

2.5.3 Wagashi

Traditional soft cheese, popularly known as *wagashi* is produced by indigenous female processors, often the wives of the nomadic Fulani herdsman, using surplus of unpasteurized milk that cannot be returned to the market for sale (Mwini & Darkwa, 2016). It is widely distributed amongst the Muslim communities, local markets and communities with natives from the northern regions of Ghana (Omore *et al.*, 2001). It is prepared by heating the milk and adding stem extracts from the Sodom apple plant (*Calotropis procera*) or *Bryophyllum* stems at the rate of 4 stems to a liter of milk. The resultant curd is scooped into a perforated calabash or a cheese cloth to assist the draining of the whey and then hand squeezed to produce firm balls of the product (Agriculture and Consumer Protection, 1990; Smallholder Dairy Network, 2006). The raw *wagashi* is stable for up to 14 days when kept in brine and just 3 days when not preserved in brine (Pantaleao and Moens, 1990). Raw *wagashi* comes in an opaque white color with small pinholes whilst the fried version has a characteristic orange to light brown color. Consumers prefer it either in its raw state to be used in their various homes as protein source in their soups or stews or in a fried state, which is considered as a RTE snack.

2.5.4 Yoghurt

Yoghurt is a well-known fermented dairy product. It is produced by culturing milk with starter cultures containing *Streptococcus thermophilus*, and *Lactobacillus delbrueckii subsp bulgaricus* (Chipurura *et al.*, 2014). Yoghurt consumption is widely appreciated because of its perceived nutritional benefits including proteins, minerals, vitamins and probiotic effect which helps the immune system (Aluko, 2017). Processing of milk into yoghurt involves key steps including milk standardization, homogenization, pasteurization, inoculation with starter cultures, incubation for

fermentation to occur, cooling of yoghurt and appropriate storage (Lee & Lucey, 2010). The figure below shows the main processing steps in yoghurt production.

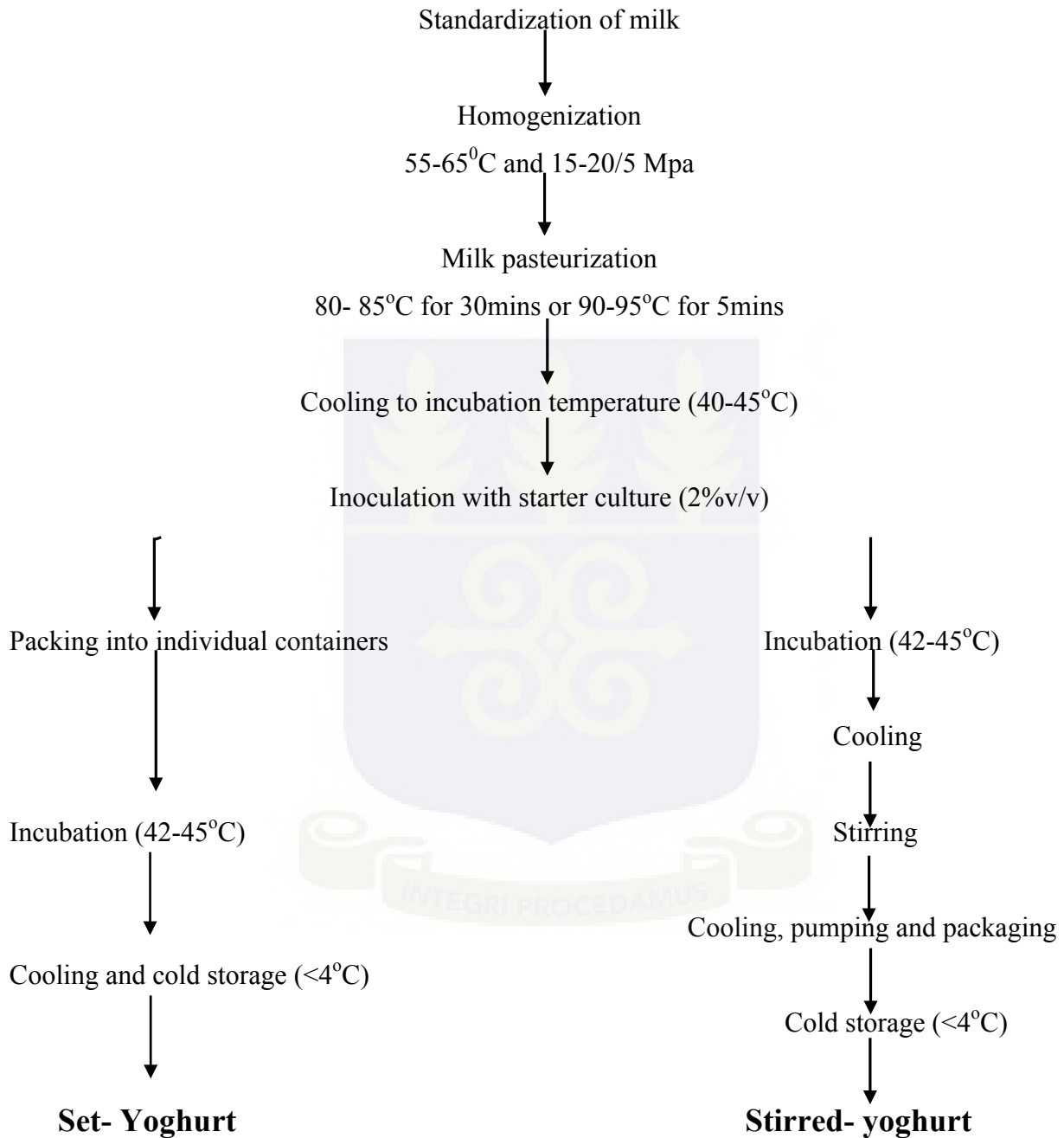


Figure 4: Yoghurt processing

Source: (Lee & Lucey, 2010)

2.6 Challenges in Milk Processing

In a broader perspective, smallholder producers who are the sole suppliers of raw milk, lack the required technological, organizational as well as the institutional capacities to effectively run the dairy business (Yilma *et al.*, 2011). Ghana's dairy industry is considered as lagging behind when it comes to world production because it lacks efficient and modern technologies which are vital not only to increase yield but to add value in terms of processing and preservation (New Food, 2016). Most dairy farmers have a subsistence way of life and in many cases, they are limited in market access because they do not meet the statutory requirements for registration and licensing as recognized processors. Also, most smallholder dairy farmers lack the necessary knowledge to produce milk to meet international standards to enable them export (New Food, 2016).

Milk is particularly prone to bacterial contamination and spoilage because of its high protein, high moisture and neutral to slightly acidic pH. This is especially true amongst smallholder dairy processors in Ghana, most of whom collect and transport milk on foot, or by bicycle or public transport often on very poor road networks (Omore *et al.*, 2004). The lack of cold chain management during transportation together with prolonged conveying times makes milk from smallholder dairy stakeholders in Ghana, prone to bacterial proliferation. Milk is reportedly safe from bacterial colonization during the first 2-3 hours after milking when the inherent antibacterial content are active and inhibit the action of the spoilage bacteria (Kurwijila, 2006). The inherent antibacterial agent breaks down and allows the spoilage bacteria to take over and multiply if cold chain is not observed after the safe period (Kurwijila, 2006).

Once more, even though *wagashi* is in a high demand, it has a very short shelf life due to the lack of adequate preservation technologies. Market women are forced to boil the *wagashi* daily which negatively affects its appearance. Studies conducted on the preservation of *wagashi* in 15% brine

solution showed an extension of the shelf life from 3 to 15 days but also affected the taste by making it too salty (Smallholder Dairy Network, 2006). Finally, smallholder dairy farmers lack flexible financial support including low interest rate loans (Knight-Jones *et al*, 2016) as well as technical advice in the form of training sessions, to help increase their production and profit yield (Kawambwa *et al*, 2014).

2.7 Milk Handling and Marketing in Ghana

Production and consumption of fresh milk in Ghana especially along the informal marketing chain has gained increased economic value due to the convenience in delivery by the farmers or assemblers, lowered prices and perceived nutritional value as compared to its processed counterparts. With all these increases in purchase and consumption of raw milk, Gidiglo (2014) investigated the milk production and marketing in Ghana, with focus on the Accra plains, and results from his preliminary survey showed that milk production in the south of Ghana was the major source of income for the herdsman who participated in his study. It was a livelihood that involved the whole family, including wives and children who had no amount of requisite formal training in the handling of fresh milk. The study also noted that less than a quarter (20%) of the population (N=150) had good training in the handling of the fresh milk. A detailed survey of street vending in Accra by FAO (2016) pointed out that 18.1% of the vendors interviewed (N= 3175) had no formal education and 38% of the vendors also had never attended or received any form of food hygiene and safety training courses. More than half of the vendors interviewed (51.2%) said health inspectors or regulators had never visited them.

The high level of ignorance together with unchecked practices by the vendors and processors in this regard is translated into their activities on site including milking, handling and storage of dairy

products. Some of these practices included the use of untreated water in the washing of milking utensils as well as milking in the kraal instead of a designated milking area. With economic gains acting as the main reason behind the sale of raw milk, it is indeed not surprising that unsold milk is subsequently used to make the local cheese known as *wagashi* (Gidiglo, 2014; Otchere & Okantah, 2001).

Many African countries including Ghana, lack standardization together with monitoring and food safety management programs like Hazard Analysis of Critical Control Points (HACCP) respectively in the sale of a highly perishable product such as fresh milk. To begin with, Ayebo Amadu (1973) in a study in the southern part of Ghana established a correlation between the various practices prior to the milking of the animals and the final bacterial load in the milk. The author arrived at this conclusion by comparing the total bacterial counts from swabs from nomadic herdsmen, the formal large scale Amrahia farms, and from the Animal Research Institute (ARI). The mean bacterial counts recorded were 345,000 cfu/ml, 27,000 cfu/ml and 61,000 cfu/ml respectively. From the comparatively higher counts observed with counts from the herdsmen, it was concluded first that, more handlers in the value chain led to poor quality of milk, and that better hygienic practices during milking resulted in better milk safety.

2.8 Milk as a Bacterial Growth Medium

Milk is a nutrient dense food encompassing principal macro and micro nutrients and naturally occurring enzymes. Comprising of 87% water and a neutral pH, milk presents an excellent medium for proliferation of microflora present (Guétouache *et al.*, 2014).

Like a mammalian gland, a healthy udder serves as a reservoir for naturally occurring useful microflora whose activities during controlled fermentation produces acidity, flavor compounds and ripening enzyme during the production of cheese. Inherent bacteria of significant impact in the dairy industry includes lactic acid bacteria, proteolytic bacteria, lipolytic bacteria and yeasts (Quigley *et al*, 2013). The moisture content, water activity, pH and high nutrient content provides a suitable environment for the microflora to proliferate when milk is not preserved soon after milking.

2.9 Contamination in milk

Milk is considered virtually sterile when secreted into an uninfected udder of the animal (Nada *et al*, 2012). Microbial contamination in milk can go a long way to cause milk-borne diseases to humans. Potential sources of microbial contamination are primarily linked to contamination from infected milking animals in a condition referred to as *mastitis* and contamination resulting from milking under unhygienic situations (Boor & Murphy, 2002).

Mastitis is a condition that leads to contamination of milk marked by an inflammation in the udder as a result of microbial infection. Other conditions such as contaminated water, feed, soil, bedding or other infected animals have been reported to be directly linked either as independent or collectively related to the diversity of the bacterial profile of raw milk (Verdier-metz, 2009).

There are many opportunities for microbial contamination and proliferation along the milk value chain. These includes direct milking by hands, milk handlers at the various stages of the value chain, poorly kept milking equipment and utensils, the use of contaminated water in the milking operation or the cleaning of the utensils involved, and the lack of maintenance of cold chain during transportation and storage (Abdelgadir *et al.*, 2014; Gonfa *et al*, 2001)

2.10 Milk-borne Infections

Over 75% of the milk marketed in developing countries are sold in their unpasteurized state through informal channels (Bertu *et al.*, 2010). Consumers are therefore at risk of contracting milk-borne diseases especially because the milk is also transported and sold under tropical temperatures ranging from 17 – 35 °C. Milk-borne infections are caused by ingestion of milk and milk products containing pathogenic microorganisms or their respective metabolites. Common milk borne infections reported include anthrax, listeriosis, salmonellosis, campylobacteriosis, brucellosis, leptospirosis, Q fever and bovine tuberculosis (Dhanashekar *et al.*, 2012). These diseases pose a serious threat to the health and overall wellbeing of the consumers as well as their economic productivity. Several cases of milk-borne human infections have however been reported in other parts of the world, the table below shows illness associated with milk reported in several industrialized countries.

Table 1 Illness Associated with Milk in Several Industrialized Countries

Agent	Country	Year	Food	Number of cases
<i>S. aureus</i>	Japan	2000	Powdered skim-milk products	13,420
<i>Salmonella enteridis</i>	France	2001	Cantal cheese made with raw milk from cow	215
<i>Listeria monocytogenes</i>	Canada	2008	Pasteurized milk cheese	38
<i>E. coli</i> O157:H7	USA (multistate)	2010	Aged raw milk Gouda cheese	41

Source: (Fusco & Quero, 2014)

Donkor *et al.* (2007) studied the prevalence of bacteria including *Yersinia*, *Escherichia coli*, *Staphylococcus* spp., *Bacillus* spp., *Mycobacteria* and *Enterobacter* spp. which are commonly associated with raw milk in Ghana. Their study focused on raw milk sold in two major cities in the southern part of Ghana. Poor hygienic conditions usually in the kraal, together with contamination by mud and dung around the milking areas were amongst the few observations made during this study. From 96 milk samples, *Yersinia* was found to be the most prevalent organism (19.8%) followed by *Klebsiella* (16.7%), *Staphylococcus* (14.6%), *Bacillus* spp. (11.5%), *Proteus* (7.3%), *Enterobacter* spp. (6.3%), *E. coli* (2.1%) and *Mycobacterium* spp. (1%). Although the observed prevalence rates were not that alarming, activities such as pooling of milk by assemblers, frequent unhygienic handling practices, together with a lack of pasteurization could worsen results further down the value chain. An earlier study by Sampene-Donkor (2002) also reported an even higher prevalence of *E. coli* (17.3%), *Yersinia* (25.9%) and *Staphylococcus* (24.1%) in milk. The poor milk quality was attributed to the fact that, 64 out of the 143 milk samples were adulterated with untreated water. In a report by Omore *et al.*, (2003), concerns were expressed about similar arguments pertaining to post-process contamination being a major key player in reported cases of contamination in boiled milk samples. After achieving a low average bacterial count of 10^4 cfu/ml due to raw milk hygiene interventions at farms, processing units and local markets in Burkina Faso, a neighboring country to Ghana, Millogo (2010) emphasized that good udder health combined with training of major stakeholders along the value chain of the raw milk industry can go a long way to assure the safety of milk.

2.11 Important Milk Pathogens

2.11.1 *Escherichia coli* O157:H7

E. coli is a facultative anaerobic gram-negative bacterium naturally present in the intestinal microflora of humans and animals. Not all strains of *E. coli* are pathogenic (New Hampshire, 2003), however some strains contain variations, in that they express virulence factors (Fusco & Quero, 2014). Pathogenic *E. coli* is indicative of fecal contamination in food or water and is broadly grouped into two major categories namely enteric pathogens and extra intestinal pathogens with symptoms ranging from diarrhea to urinary tract infections, respectively.

The prevalence of *E. coli* in milk and dairy environment has been found to be low in the recent past in Ghana. In a study carried out in the coastal savannah of Ghana (Addo *et al.*, 2010) 250 milk samples were examined for the prevalence of this pathogen. It was found that, 11.5% out of 66.0% of the coliform population were indeed *E. coli*, however, none of them tested positive for the *E. coli* O157:H7. In the northern part of Ghana also, an independent study conducted to examine the prevalence of shiga toxin producing *E. coli* in both raw milk and cattle feces (Saba *et al.*, 2015) revealed that, out of a total of 150 raw milk samples used for the study, 74 samples representing 49.3% contained *E. coli*. This result duly expressed an indication of fecal matter in the product but none of the isolates obtained was positive for *E. coli* O157:H7. This result was also in line with the low prevalence of *E. coli* O157:H7 that had been expressed in a similar study in Tanzania (Swai and Schoonman 2011), where none presumptively identified *E. coli* isolates were confirmed as *E. coli* O157:H7. On the other hand Saba *et al.*, (2015) indicated that out of the 55 fecal materials sampled from milking cows that tested positive for shiga toxin *E. coli*, 7 samples (12.7%) were suspected to be *E. coli* O157:H7. In southern Ghana however, Omoro *et al.* (2004) showed that

out of 419 milk samples sampled across both wet and dry seasons, only 3 samples tested positive for *E. coli* O157:H7.

2.11.2 *Listeria monocytogenes*

L. monocytogenes is a global concern, ubiquitous, gram positive, facultative anaerobe foodborne pathogen that causes a high fatality rate especially amongst the high-risk groups including elderly and immune-compromised as well as pregnant women (Garcia & Heredia, 2009). Listeriosis is a severe disease caused by exposure to a typical *L. monocytogenes* contamination level of 10^6 and as low as 10^3 . Symptoms of this infection includes late term spontaneous abortion, prenatal infection, meningitis, encephalitis, septicemia and gastroenteritis (Garcia & Heredia, 2009).

The direct implications of milk borne infections had not received enough recognition in Ghana until Appiah (2012) commenced his comprehensive study on *L. monocytogenes* in milk sold in Ashiaman, a suburb of Tema in Ghana. The author reported that high incidence of premature abortions at the Tema general hospital, the main hospital for the Tema metropolitan area, motivated his study. He suspected *L. monocytogenes* to have been playing a key role in the incidence since the raw milk together with other milk products including the local cheese was highly consumed in that community. As part of the study, a survey conducted in the municipality revealed 55 female consumers, representing 66.3% of the sampled population, had experienced spontaneous abortions. Also, 14 of these women representing 29.8% reported having gone through the experience twice. Six female consumers who participated in the study however said they had also experienced stillbirth. His study revealed a high proportion of the samples (47%) including raw, retailed, boiled and fermented milk sold on the informal markets were contaminated with *L. monocytogenes*. The levels of contamination were analyzed in separate stages including on farm

(42%), after transport prior to boiling (79%) and after boiling (18%). The act of bulking milk or pooling milk from different sources, the use of plastic containers, which are difficult to clean, and travelling long distances with milk without maintenance of cold chain, were identified as the factors that possibly contributed to the growth of pathogen in milk. Inadequate heating-to-time fraction and post contamination practices were also reasons assigned to the high prevalence observed.

2.11.3 *Staphylococcus aureus*

The genus *Staphylococcus* is broadly categorized by their ability to produce coagulase, hence the coagulase positive and coagulase negative forms of *Staphylococcus*. The bacteria are known to easily grow and establish themselves as commensals on the skin and nostrils of warm blooded animals and humans (Costa *et al*, 2013). *S. aureus* is a facultative anaerobic coagulase, catalase positive and Gram-positive coccus. It regarded as the most pathogenic species of the genus *Staphylococcus* and can grow in a wide variety of foods considering its ability to grow in a wide range of temperatures (7 – 48.5), a wide pH range of 4.2 – 9.3 and salt concentration of up to 15% (Loir *et al*, 2003).

Staphylococcal food poisoning is evident after ingestion of approximately between 10^5 to 10^8 of the enterotoxin produced by the *S. aureus*. Symptoms range from nausea, vomiting and acute illnesses including sweating, headache and dehydration to extreme conditions including blood and mucus in feces (Garcia & Heredia, 2009) and toxic shock syndrome (Fusco *et al*, 2011)

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study design and Sampling sites

The study made use of culture based methods, biochemical methods and molecular methods for microbiological analysis. It examined the microbiological quality of milk products sold directly by the processors and the retailers in selected informal markets and used molecular tools for confirmation of pathogens. The chosen markets were selected due to the fact that sampled products were mostly produced and consumed within the locality. The sampling sites and products sampled were selected based on availability and popularity amongst the target consumer group in Ghana. A total of 117 samples were used during this study. *Brukina*, *nunu*, raw *wagashi*, fried *wagashi* and boiled milk were sampled on two different occasions from the chosen sites namely, Ashiaman, Tulaku, Nima, Newtown and Madina markets. Commercial yoghurts were sampled from Shoprite and Game from the Accra shopping mall, Max mart (A&C shopping mall), 37 Max mart shopping mall and All Needs supermarket in Accra metropolis. Raw milk was also sampled based on availability from selected nomadic Fulani herdsmen from Oyibi, Abokobi and Bawaleshi, near Dodowa. Processors and retailers were made to conveniently serve the samples in the same manner they would have handled them when serving the customers or consumers.

3.2 Sampling for microbiological analysis

A convenient sampling approach was used during this study. Samples were obtained from available retailers and stationary known processors within each sampling site. Samples were collected into sterile bags, appropriately labelled with respective codes and transported in thermos

ice chests disinfected with 70% ethanol. All samples were transported on ice and analyzed in the laboratory within two hours of sampling.

Table 2 Sampling Sites and respective samples obtained for microbiological analysis

Sampling Site	Product						
	<i>Nunu</i>	<i>Brukina</i>	Raw milk	Boiled milk	Raw <i>wagashi</i>	Fried <i>wagashi</i>	Yoghurt
Nima	1	5	0	6	3	3	0
Ashiaman	2	4	0	6	3	3	0
Tulaku	2	4	0	6	3	3	0
Newtown	0	6	0	0	4	2	0
Madina	3	3	0	0	4	2	0
Oyibi	0	0	5	0	0	0	0
Abokobi	0	0	5	0	0	0	0
Bawaleshi	0	0	5	0	0	0	0
Shopping mall	0	0	0	0	0	0	24
Total	8	22	15	18	17	13	24

3.2.1 Sampling from wholesalers / retailers

Milk samples were purchased from wholesalers or what could be referred to as assemblers and retailers from the selected informal markets.

3.2.2 Sampling from milk processors

Sampling was done at the processing site. Where it was available, samples of boiled milk intended for processing were also collected. Samples were purchased in original packaged form and sealed in sterile stomacher bags for transport on ice to the laboratory.

3.3 Laboratory microbiological analysis

The milk samples were analyzed for the presence of total coliforms, fecal coliforms, *E. coli*, *Staphylococci*, and *L. monocytogenes*.

3.3.1 Sample preparation

Ten milliliters of liquid milk sample and 10 g of solid sample were aseptically measured into sterile stomacher bags with the addition of 90 mL sterile peptone water. The weighed samples were homogenized for 2 minutes in a Seward stomacher blender. Serial dilutions for the various microbial counts earlier mentioned were then carried out as described by the International Commission for Microbiological Specifications for Foods (Zwietering et al., 2008).

3.3.2 Enumeration of Total *Staphylococci* and Detection of *S. aureus*

Baird-Parker agar (Oxoid CM275) with egg yolk tellurite emulsion supplement (Oxoid) was used in a pour plating method for three serial dilutions of the samples. After incubation at 35-37°C for up to 48 hours, plates containing 20-200 typical staphylococcal colonies (black, circular) were counted. Up to 5 colonies on each plate were subcultured on nutrient agar (Oxoid CM003) and tested for catalase activity. Colonies that were catalase positive, circular, convex, grey-black to jet-black with an off- white margin were considered presumptive for *S. aureus*.

3.3.3 Enumeration and detection of fecal coliforms and *E. coli*

Three serial decimal dilutions of the milk-peptone water homogenate were pour plated on Levine

Eosin Methylene Blue (EMB) agar (Oxoid CM0069) and incubated at 35°C for 24 hours. Plates with 20-200 Purple colonies were counted and selected colonies sub-cultured on MacConkey agar (Oxoid) at 30°C for 24 hours. Fecal coliforms fermented lactose (pale pink color on MacConkey) and were gram negative rods. *E. coli* colonies appeared circular, dry and flat with a metallic sheen. Five of such colonies were sub-cultured on Sorbitol MacConkey (SMAC) agar (Merck), where they appeared as pin point, pale pink colonies. These suspect colonies were then purified on nutrient agar and then transferred unto Triple sugar iron agar (TSI) slants, Simon's citrate slants and Sulphur-Indole-Motility (SIM) agar. *E. coli* colonies were indole positive, CO₂ positive, H₂S negative, citrate negative and could ferment glucose and sucrose.

3.3.4 Detection of *Listeria* species

A 1 in 10 dilution of milk homogenate with half strength Fraser broth was incubated for 24 hours at 30°C. A volume of 0.1 ml of the incubated homogenate was sub-cultured into 10 ml full strength Fraser broth and further incubated at 30°C for 26±2 hours. A loop full of the enriched homogenate was then streaked unto well-dried plates of Chromogenic *Listeria* agar (LCA, Oxoid CM1017), which was incubated at 35±2°C for 24 hours or until growth was satisfactory. Suspect *Listeria* colonies appeared as blue-green colonies surrounded by an opaque halo. Visible colonies on each of the plates were further sub-cultured onto separate nutrient agar and sheep blood agar plates. Confirmation of the colonies was done with MALDI-TOF.

3.4 Confirmation of Pathogens Using Quantitative Polymerase Chain Reaction (qPCR)

Three out of the six different fresh milk products, namely *brukina*, *nunu* and boiled milk analyzed were subjected to qPCR to confirm the prevalence of the *E. coli* O157:H7, *L. monocytogenes* and *S. aureus*.

3.4.1 DNA Extraction

Frozen milk samples (-20°C) were thawed at 4°C for 3 hours prior to the extraction. Ten milliliters of the milk was aseptically measured into a sterile stomacher bag with the addition of 90 ml of pre-warmed (45°C) 20% sodium citrate. The mixture was then homogenized with a stomach for 5 minutes. Ten milliliters of the homogenate was then centrifuged at 12,000 g for 10 minutes at 20°C. The pellet obtained was further treated with 1ml of sodium citrate in a 1.5 ml centrifuge tube and centrifuged at 11,000 rpm at 20°C for 5 minutes. Autoclaved swabs were used to clean any visible suspension created due to the centrifugation step. The extraction protocol employed was provided by the MoBIO kits manufacturers with a few modifications (extra centrifuge at 10,000 g for 30 seconds before adding the MD4 reagent and secondly, elution with 50ul molecular grade water). DNA was quantified using Nano Drop spectrophotometer, and placed under -20°C storage (Rasolofo *et al*, 2010).

3.4.2 Quantitative Polymerase Chain Reaction

The DNA obtained from the milk samples were used to conduct specific detection of *E. coli* O157:H7, *L. monocytogenes* and *S. aureus* using specific primers in a 96-well block uniplex RT - PCR using Viia 7 system by Applied Biosystems.

The process made use of a total reaction volume of 20 ul, comprising of 10 ul of 100 uM SYBR

Green mix (1x), 0.8 ul each of the designated primers with a concentration of 400 nM, 5 ul of DNA with a 1:10 dilution factor, 3 ul of molecular grade water and 0.4 ul of 0.2 mg BSA (Bovine Serum Albumin) at a reaction melting temperature of 60°C. Five micro liters of water was used as negative control in the reaction.

3.4.2.1 Design of Primers

The sensitivity and specificity of the primers for *E. coli* O157:H7 and *S. aureus* were established by Aznar & Elizaqui (2008) whilst that of *L. monocytogenes* was done by Rodríguez-la *et al.*, (2004). For this study, the primers used were designed by Eurofin Genomics (12701 Plantside Dr, Louisville KY, 40204). Stock primers were prepared by addition of appropriate volumes of molecular grade water as stated by the manufacturer to attain a final concentration of 100uM. Prior to the experiment, the BLAST-N tool was used to confirm that none of the selected primer sequences recognized any other registered DNA sequence apart its target sequence. The selected primers for the detection of *E. coli* O157:H7 were designed to detect specific fragments in *uidA* gene which encodes for an enzyme known as beta –glucuronidase in *E. coli* O157:H7.

The primers selected for the detection for of *L. monocytogenes* were designed to focus on the detection of specific fragments in the *hly* gene which is responsible for encoding listeriolysin O, an essential virulence factor in *L. monocytogenes*. The primers for the detection of *S. aureus* was designed to detect specific fragments in the *nuc* gene, which is known to have a high correlation with the production of enterotoxins and encodes for the thermostable nuclease enzyme in *S. aureus*.

Table 3 Primers used for amplification of virulence genes *uidA*, *hly* and *nuc* in uniplex qPCR

Target	Primer	Organism	T.(°C)	Sequence (5' – 3')
<i>uidA</i>	uidAR383	<i>E. coli</i> O157:H7	64	ACC AGA CGT TGC CCA CAT AAT T
	uidAF241	<i>E. coli</i> O157:H7	64	CAG TCT GGA TCG CGA AAA CTG
				Source: (Aznar & Elizaqui, 2008)
<i>hly</i>	hlyQF	<i>L. monocytogenes</i>	63	CAT GGC ACC ACC AGC ATC T
	hlyQR	<i>L. monocytogenes</i>	63	ATC CGC GTG TTT CTT TC GA
				Source: (Rodríguez-la et al., 2004)
<i>nuc</i>	R465	<i>S. aureus</i>	76	TCG ACT ATA TAC TGT TGG ATC TTC AGAA
	F5	<i>S. aureus</i>	88	CGC TAC TAG TTG CTT AGTGTT AAC TTT AGT TG
				Source: (Aznar & Elizaqui, 2008)

3.5 Control Strains

The control strains used for this study were obtained from the Canadian Research Institute for Food Safety (CRIFS) culture bank at the University of Guelph. The *E. coli* O157:H7 positive control was a bioluminescent strain (luminescent EHEC constructs (LEE 1::luxCDABE), grown in Luria-Bertani (Fisher Scientific) broth supplemented with ampicillin (AMP) and kanamycin (KM), each at a concentration of 50 ug/ml. The *L. monocytogenes* positive control strain used was an outbreak strain isolated from sliced turkey (2000), serotype 1/2a, Cornell ID FSL F6-154.

3.6 Viability Test

Selected RTE dairy samples that tested positive for targeted pathogens (*E. coli* O157:H7, *L. monocytogenes* and *S. aureus*) using qPCR, were further subjected to selective enrichment and plating to determine the viability of the pathogens in the samples for specific detection purposes.

3.6.1 Detection of *L. monocytogenes*

Ten milliliters of the milk sample was homogenized in a pre-warmed (30°C) 90 ml Listeria enrichment broth in a sterile stomacher bag and incubated at 30 °C for 48 hours. Ten milliliter of modified Fraser broth was inoculated with 0.1ml of the enriched sample and incubated at 35°C for 24 hours. A loop of a positively enriched sample was streaked onto dried Oxford (OXA) plates and modified oxford (MOX) plates. A positive broth had a characteristic darkened to black, dark brown or dark green color whilst a negative broth had a straw like. The negative broths were reincubated for 24 hours after which it was streaked on both Oxford agar and Modified Oxford agar if positive. Distinct colonies on both Oxford and modified Oxford agars suspected to be *Listeria* were viewed as black colonies surrounded by black haloes after 24hours. Presumptive *Listeria* species were confirmed using the API.

3.6.2 Detection of *E. coli* O157:H7

Ten milliliters of the milk sample was homogenized in a pre-warmed (30°C) 90 ml Brain Heart Infusion (BHI) broth in a stomacher bag. The homogenate was allowed to settle for 10 minutes at room temperature and aseptically transferred into a sterile tube and incubated of 3 hours at 35°C. The enriched content was transferred into 90 ml double strength Tryptone Phosphate broth and incubated for 20 hours at 44°C. Contents were streaked onto Sorbitol MacConkey (SMAC) plates and incubated at 35°C for 20hours. Typical colonies on the MacConkey plates appeared with a distinct brick red color.

3.6.3 Detection of *S. aureus*

Ten milliliters of the milk sample was homogenized in a pre-warmed (30 °C) 90 ml Brain Heart Infusion (BHI) broth in a stomacher bag. The homogenate was allowed to settle for 10 minutes at room temperature and aseptically transferred into a sterile tube and incubated for 3 hours at 35°C. The enriched sample was plated on Baird Parker agar plates containing egg yolk tellurite emulsion supplement. Typical *S. aureus* appeared as black colonies with transparent hollows surrounding it.



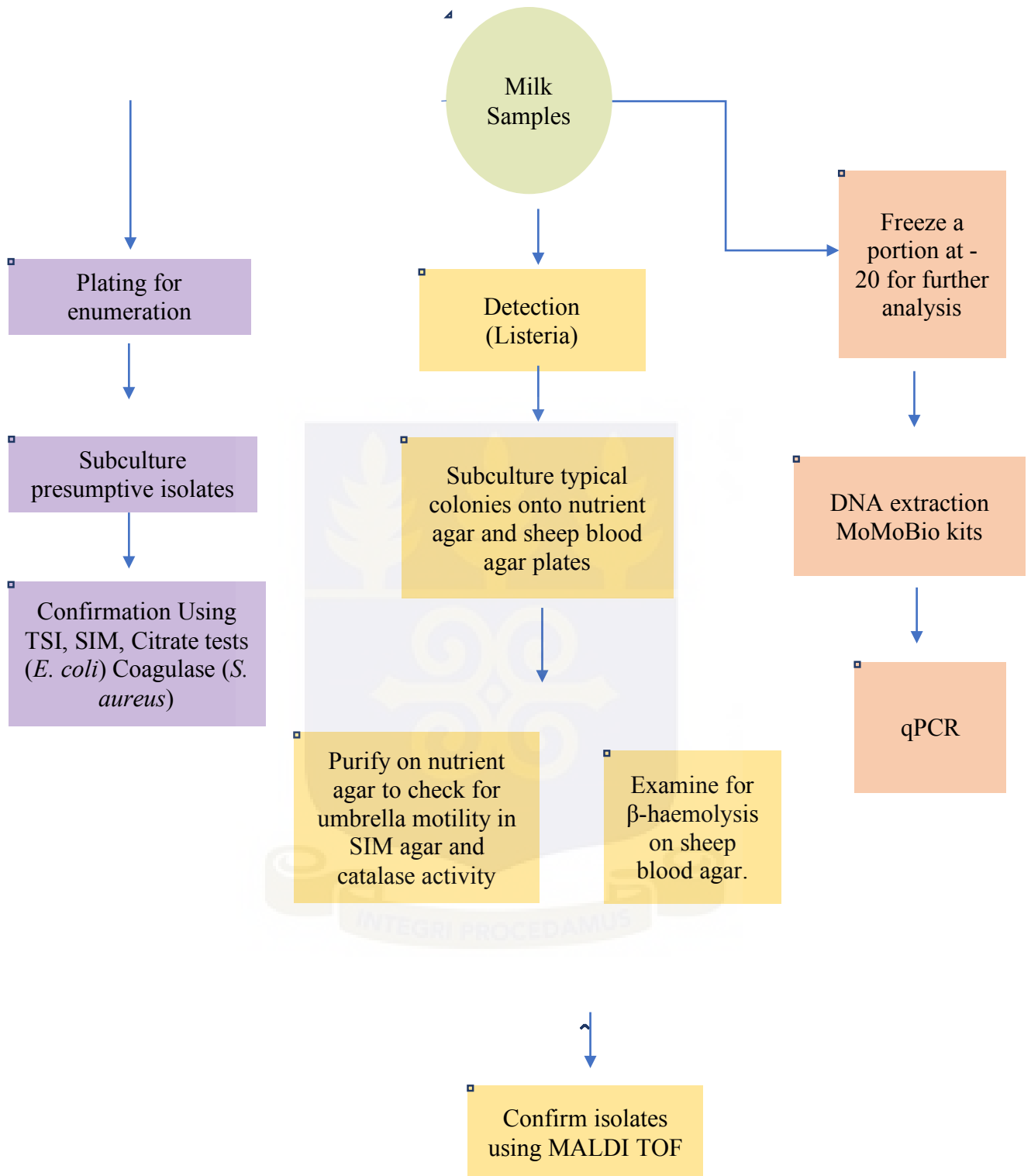


Figure 5 : Layout of microbiological analysis

3.7 Statistical Analysis

A set of descriptive statistics including means, standard deviations, and frequencies were used to analyze microbial counts. One-way Analysis of variance (ANOVA) was used to assess the significance of differences between counts of different products from each market at $P \leq 0.05$. The prevalence of isolated microorganisms was determined using Cross tabulations. All statistical analysis was done with IBM SPSS version 21 and Microsoft Excel (2016)



CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Microbiological and Hygienic Quality of RTE Dairy Products

The hygienic quality of the samples was analyzed using coliform and staphylococci counts as indices. Their presence in milk and milk products signifies possible fecal contamination and poor handling practices along the milk value chain. Out of a total of 117 samples, 79 were contaminated with coliforms, representing 68%. The lowest incidence of coliform was found in fried *wagashi*, while raw *wagashi* recorded the highest occurrence of coliforms. Table 4 is the summary of the mean counts of total coliforms, fecal coliforms, *E. coli* and Staphylococci in the different milk products sampled in the study.

Table 4 Mean counts of hygienic and quality indicators in fresh milk and fresh milk products

Milk Product	Mean \pm Standard Deviation (Log cfu/ml)			
	Total coliforms	Fecal coliforms	<i>E. coli</i>	<i>Staphylococcus</i>
Raw Milk	3.6413 \pm 2.5645 ^{abc}	2.6077 \pm 1.8589 ^{ab}	2.1072 \pm 2.1208 ^{ab}	3.0986 \pm 1.0866 ^{ab}
Boiled Milk	3.6127 \pm 3.0650 ^b	2.4771 \pm 2.3568 ^{bc}	1.4082 \pm 2.0979 ^b	1.9367 \pm 2.2502 ^b
<i>Nunu</i>	2.8497 \pm 3.0933 ^b	2.1099 \pm 2.2830 ^b	1.9513 \pm 2.1085 ^b	1.8575 \pm 2.1093 ^b
<i>Brukina</i>	4.4201 \pm 2.8466 ^{cd}	2.6573 \pm 2.0528 ^{cd}	2.1611 \pm 2.0100 ^{cd}	2.7465 \pm 1.2507 ^{cd}
Raw <i>wagashi</i>	5.9039 \pm 3.7874 ^{ccc}	4.0804 \pm 2.8043 ^{cef}	2.6147 \pm 2.6514 ^{ccc}	2.2496 \pm 1.7692 ^{ccc}
Fried <i>wagashi</i>	0.4000 \pm 1.0583 ^{ab}	0.0000 \pm 0.0000 ^{ab}	0.0000 \pm 0.0000 ^{ab}	0.0000 \pm 0.0000 ^{ab}
Yoghurt	1.1812 \pm 1.8852 ^{abc}	0.3450 \pm 0.9374 ^{abef}	0.2470 \pm 0.7039 ^{abc}	0.9170 \pm 1.3891 ^{abc}

Values in the same column with different superscripts are significantly different at $\alpha < 0.05$

The highest mean coliform and *E. coli* counts were detected in raw *wagashi*. This was unsurprising considering the fact that, it had received the least amount of heat treatment, among all the dairy products tested. Occasionally, raw *wagashi* undergoes further processing in the form of either deep-frying or cooking, which subsequently kills all vegetative bacteria present. This was evident in the significantly lower mean coliform counts found in the fried *wagashi*, which also happened to be the lowest mean coliform count of all the products tested. The high counts found in the raw *wagashi* were unacceptable and could be traced to a few unregulated practices involved in the making of the product. For example, the coagulant used in the making of the cheese is crushed to obtain the sap using stones and other sharp objects mostly on unsanitary surfaces. Also, the mixture of the milk and coagulant is sifted using bare hands, which could be potential sources of contamination. Again, during the draining of the whey, the cheese is often left out in the open without lids, exposing it to flies and other contaminants in the environment. Other observation made which could possibly account for the high counts of coliforms and *E. coli* observed in the other dairy products included milk vendors handling money and waste containers concurrently. All unsold *wagashi* were also returned to the market the following day in the same plastic containers with brine solution, with no additional heating or cooling.

Figures 5 and 6 are pictures depicting the retail and storage of raw *wagashi* in the local informal markets in Ghana.



Figure 6: Retail of *wagashi* in local informal milk market



Figure 7: Storage of raw *wagashi* in local informal markets

The use of plastic containers for milk storage according to Swai & Schoonman (2011) enables coliforms to build up in the form of biofilms in the moist residues which could then contaminate subsequent batches of milk when not properly sanitized. Additionally, unhygienic practices including milking in kraals, not sanitizing the teats before and after milking, may have contributed to the contamination with coliforms. Figures 8 and 9 shows the fecal coliform content (FCC) and total coliform content (TCC) in RTE dairy products analysed.

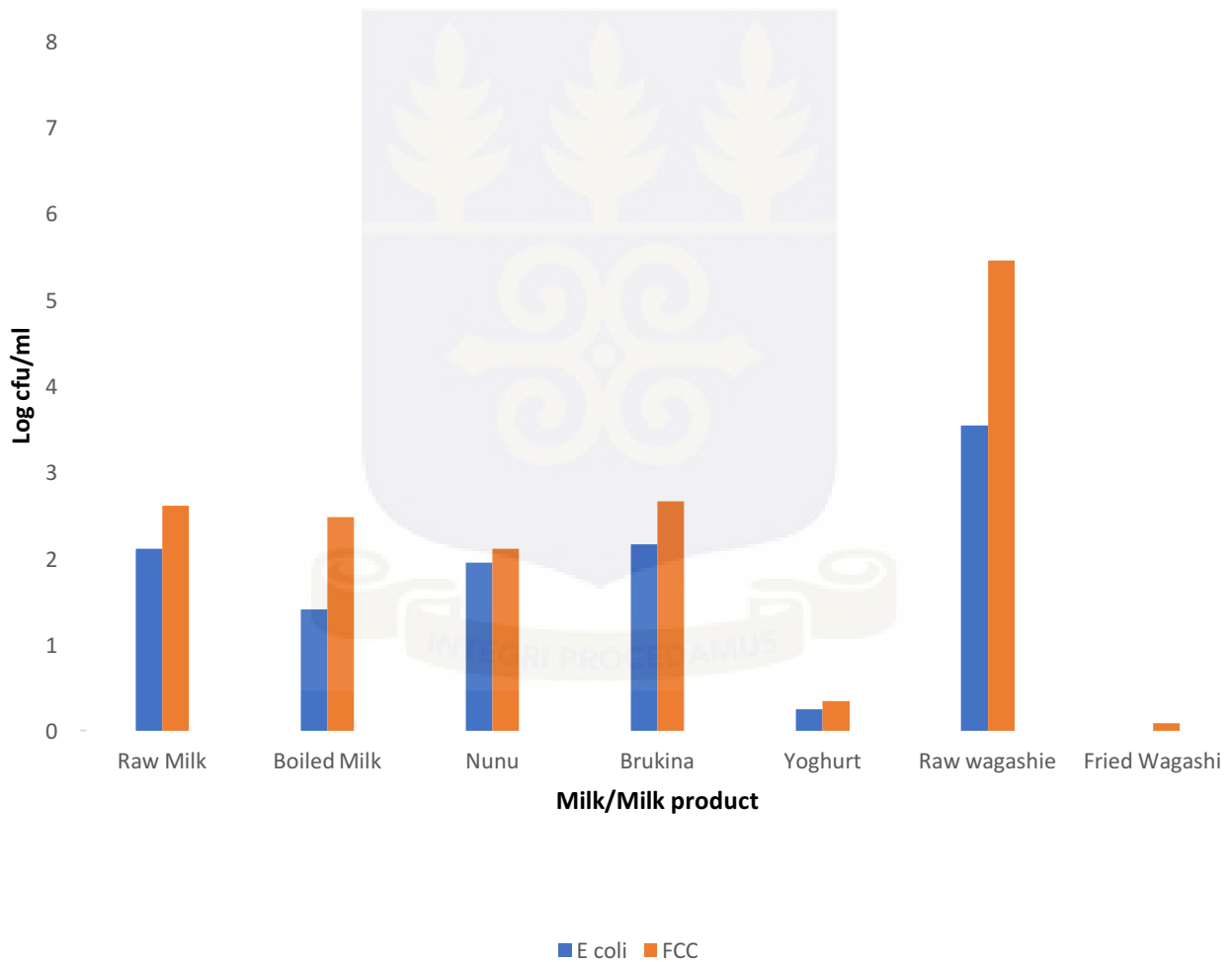


Figure 8: Fecal coliform count of dairy products

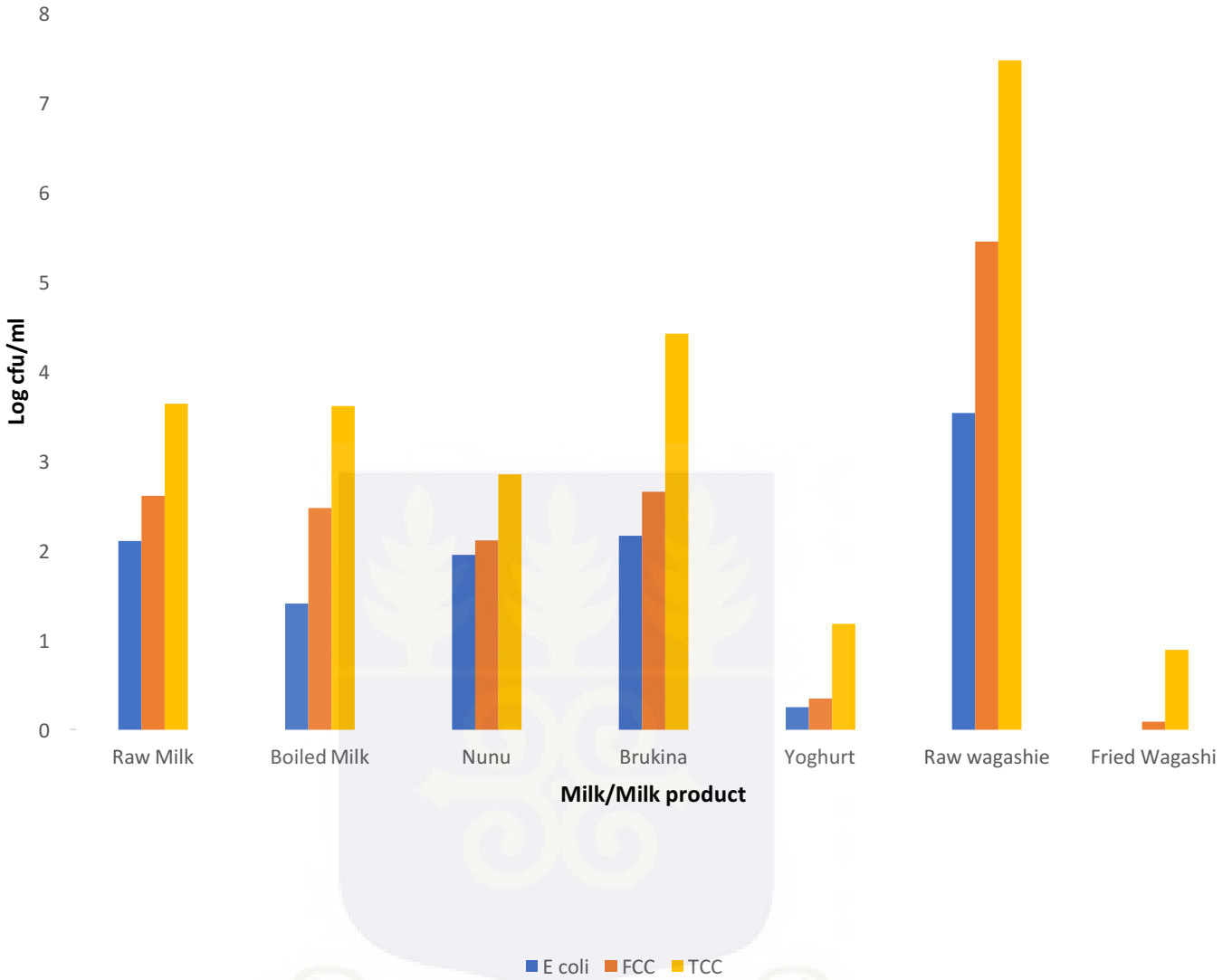
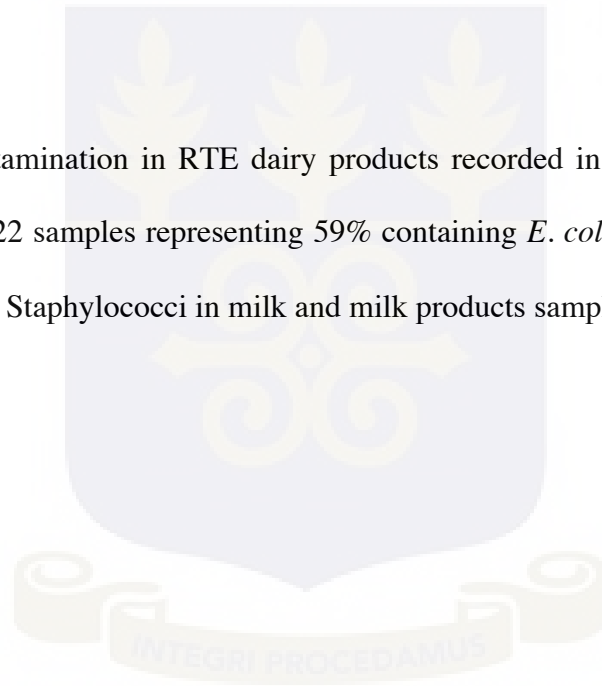


Figure 9: Total coliform counts of dairy products

The yoghurt samples analyzed recorded a relatively lower coliform count as compared to the other fresh milk samples implicated in coliform contamination. Dirisu *et al*(2015) reported similar coliform concentration whilst Aluku (2017) reported no detection of coliforms. This is because the active lactic acid bacteria included in the starter culture used in the production of yoghurt suppresses the growth of enterobacteraceae present in the milk flora (Soomro *et al.*, 2002).

A similar fermented milk product, *nunu*, which is spontaneously fermented, recorded higher coliform content than yoghurt. This could be as a result of low acid content, due to insufficient fermentation. Akabanda *et al.* (2010) reported that a reduction in the enterobacteriaceae population in *nunu* from 5.19 ± 0.63 cfu/ml at 0hrs to 0.00 ± 0.00 cfu/ml after 48 hours of fermentation, suggesting the active role of low pH caused by fermentation in suppressing the growth of enterobacteriaceae present. Other possible causes of contamination in *nunu* could arise from back slopping employed during the commercial production or cross contamination from the utensils used.

The highest *E. coli* contamination in RTE dairy products recorded in this study was found in *brukina*, with 13 out of 22 samples representing 59% containing *E. coli*. Figure 9 represents the prevalence of *E. coli* and Staphylococci in milk and milk products sampled in the study.



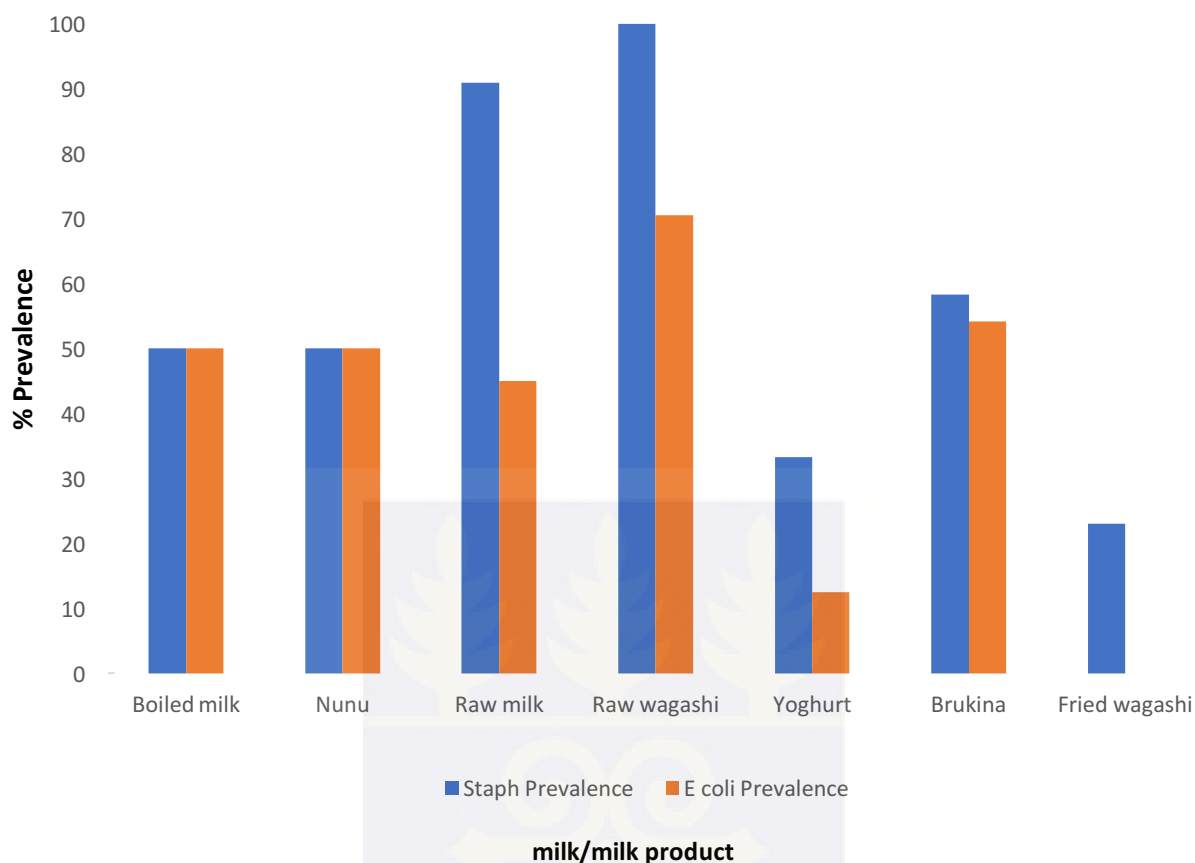


Figure 10: Prevalence of *E. coli* and Staphylococci in the fresh milk and fresh milk products tested

Owing to the fact that most of the samples were already processed, possibly insufficient processing and post contamination could account for the generally high prevalence of *E. coli* observed. As a processed RTE milk product, the prevalence of *E. coli* in *brukina* was alarming. Even though the results obtained from this study was lower than what Tawiah (2015) reported, both studies recorded their highest prevalence in the Nima – Maamobi communities. This requires immediate attention, especially since 80% of the *brukina* consumed in Accra is produced in Nima (Ghana News Agency, 2013). *E. coli* is considered an opportunistic organism and a reliable indicator of fecal contamination and other enteropathogenic organisms (Ashbolt *et al.*, 2001). Considering the

processing of *brukina*, the high counts of *E. coli* and coliforms found in “*brukina*” may be attributed to a few unhygienic activities and insufficient processing along the production chain.

Considering that the milk used in the preparation of *brukina* is boiled, the presence of a fecal indicator in the final product could be due to reasons including the result of poor hygienic processing, not observing the required temperature-time combination during the heat treatment of the milk or a possibility of post-processing contamination. It is noteworthy to consider the possible risk of contamination in practicing the use of previously fermented milk in back slopping. The presence of millet in the production of *brukina* can also be source of coliform contamination. This can explain the slight higher coliform count in *brukina* than its fermented milk base, *nunu*. In a study by Baidoo (2017), the difference in the total coliform content of the fermented milk (0.94 ± 1.98 Log cfu/ml) and the final *brukina* product (2.91 ± 3.75 Log cfu/ml) gave a reason to suggest the role millet plays in the overall coliform content of the product. One of the key findings of the study was that, there was no significant statistical difference in coliform counts between the soaked millet (2.77 ± 2.54 Log cfu/g) and the steamed cooked millet (0.93 ± 2.01 Log cfu/g) used in the commercial processing of *brukina*. This in effect, according the study suggested the inefficiency of the steam to destroy the vegetative microorganisms present. Retail operations of *brukina* in some markets can contribute to post-process contamination. At the point of sampling, some vendors sell the constituents, that is, the milk and the millet, separately (Figure 11) and only mixed upon the request of the consumer. In such cases, the same cup and spoon were used to fetch the ingredients for each customer. The vendors also did not wear any protective clothing including gloves or hairnets, and during retail, aerosols or the soil from the dusty roads could easily contaminate the product.



Figure 11: Separate retailing of *brukina* in local informal market

The statistical similarity of counts between boiled and raw milk encountered in this study was undoubtedly, a clear indication of a shortfall in the heat treatment as a form of processing in the boiled milk. It could also suggest post-processing contamination during retailing. Classical batch pasteurization is characterized by heating milk to 63°C and holding it at that temperature for 30 minutes. The predetermined temperature and time combination is enough to destroy all vegetative bacteria present. A significant practice by milk vendors in informal local milk market was the heat treatment used in treating their milk. Figures 12 and 13 are depictions of the heat treatment and retail of milk in local fresh milk market.



Figure 1: Heat treatment and retail of boiled milk in local informal market

The milk was typically boiled on a charcoal fuelled-fire and in some cases, the milk was sold while still on fire, but without time and temperature regulation. The milk was sold to different consumers directly by using a funnel and small plastic cup without any gloves in sight. The pan containing the milk occasionally was left open which exposes the content to subsequent environmental contaminations including aerosols and soil particles from the dusty roads.



Figure 2: Retail of boiled milk in local informal markets

Consumers perceive boiled milk as safer than raw milk due to the role of heat treatment in destroying vegetative microorganisms. According to this study, boiled milk was as contaminated as raw milk. In the case of the boiled milk, the evidence of sale of milk whilst still on fire suggests lack of predetermined time and temperature combination and this can explain the coliform concentration encountered.

There was a generally high prevalence of staphylococci in the milk and milk products sampled which confirms the handling deficiencies earlier outlined. This finding agrees with findings by Mensah *et al.* (2002), which also indicated a high occurrence of *S. aureus* in street vended foods in Accra. The overall staphylococci prevalence indicated that the unhygienic handling problem was a prominent issue with all the respective markets and this may again be explained by the fact that multiple intermediaries existed in-between the producer and the final consumer of the product. A study by Omore *et al.* (2004) indicated clearly that poor-quality milk was associated with the unmonitored large-scale dairy market agents, and the large numbers of intermediaries along the

value chain which inevitably increased exposure and contamination. Most of the Staphylococci detected were found in *brukina* and raw *wagashi*. The counts for staphylococci in the milk and milk products were however, commendable given that the counts were within the acceptable limits of 3.0 Log cfu/ml/g specified by the Ghana Standards Authority (GS 955). Garcia & Heredia, (2009), reported that, ingestion of food contaminated with staphylococcus enterotoxin in concentrations between 10^5 – 10^8 is adequate to cause staphylococcus food poisoning marked with symptoms including blood and mucus in stools. The lower concentration of staphylococci may not have however, rendered the milk products entirely safe especially because staphylococcal enterotoxins associated with *S. aureus* have been found to be thermostable, which ultimately means they can survive cooking temperatures (Dhanashekar *et al.*, 2012).

4.2 Prevalence of *E. coli* O157:H7, *L. monocytogenes* and *S. aureus* based on Molecular Methods

Brukina, *nunu* and boiled milk were subjected to further molecular analysis to investigate the prevalence of the *E. coli* O157:H7, *L. monocytogenes* and *S. aureus*. The reason for the choice of these products out of the rest was because they were processed and RTE dairy products. Even though raw *wagashi* was constantly showing higher microbial counts using the cultural methods, it is rarely consumed in its raw state.

The cycle threshold values from the ViiA 7 qPCR system for *E. coli* O157:H7, *L. monocytogenes* and *S. aureus* are presented in Figure 13. This explains the abundance of the target genes *uiA*, *hly*, and *nuc* representing *E. coli* O157:H7, *L. monocytogenes* and *S. aureus* respectively in the RTE products analyzed.

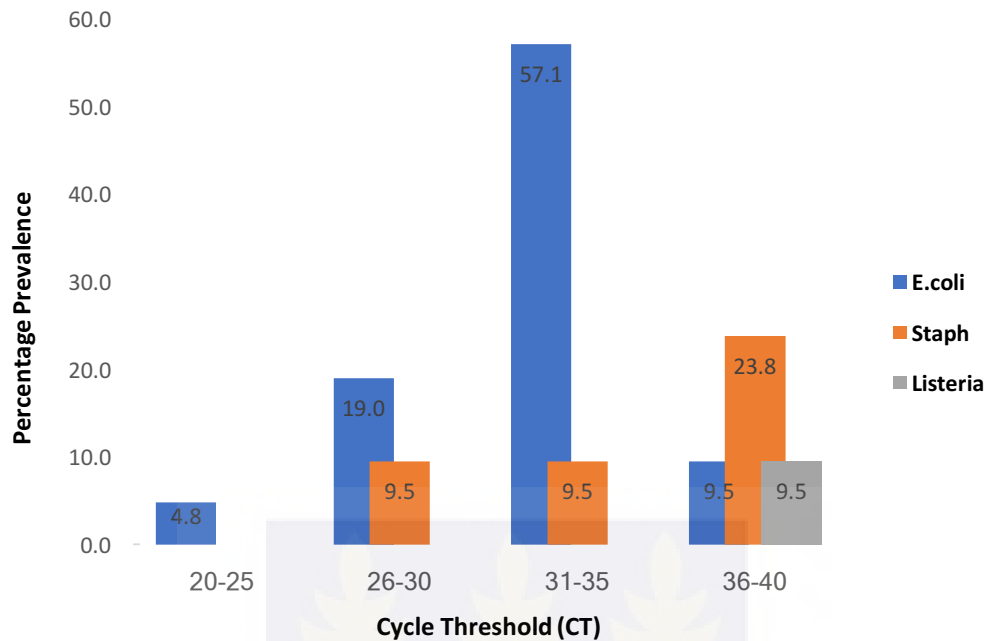


Figure 3 Cycle threshold values for target genes *uid*, *hly* and *nuc* by the Viia 7 System from boiled milk, *nunu* and *brukina* samples.

With a total of 40 cycles, the cycle threshold values obtained during this study ranged between 11.49 – 37.76, with most of the target genes detected between the 30th – 40th cycle. With a system of high resolution, capable of detecting as small as 1.5-fold changes in target quantities and a sensitivity as low as 1 copy number of target, the cycle thresholds from the study might suggest low prevalence in the samples, but it is important to take into consideration the starting volume of the original sample used and the fact that the samples were not enriched. Cooley *et al.* (2013) suggested that CT values less than 27 for a target gene from an enriched sample have a higher probability to a positive isolate upon culturing in appropriate media. Higher CT values however are not indicators of false positive, they reflect the difficulty in detecting the target gene due to either small copy number or due to the presence of other microflora especially from food matrices and environmental samples.

The CT values associated with the detection of the *hly* gene for *L. monocytogenes* (37.4, 37.7) although considered low, resulting from several conditions including possible initial minimal copy number of target nucleic acid material, it is still considered a positive reaction. This is because *L. monocytogenes* has the ability to survive harsh growth conditions including possible growth at pH of 4.7, 10% salt content and at refrigerating temperatures (Leong *et al.*, 2016).

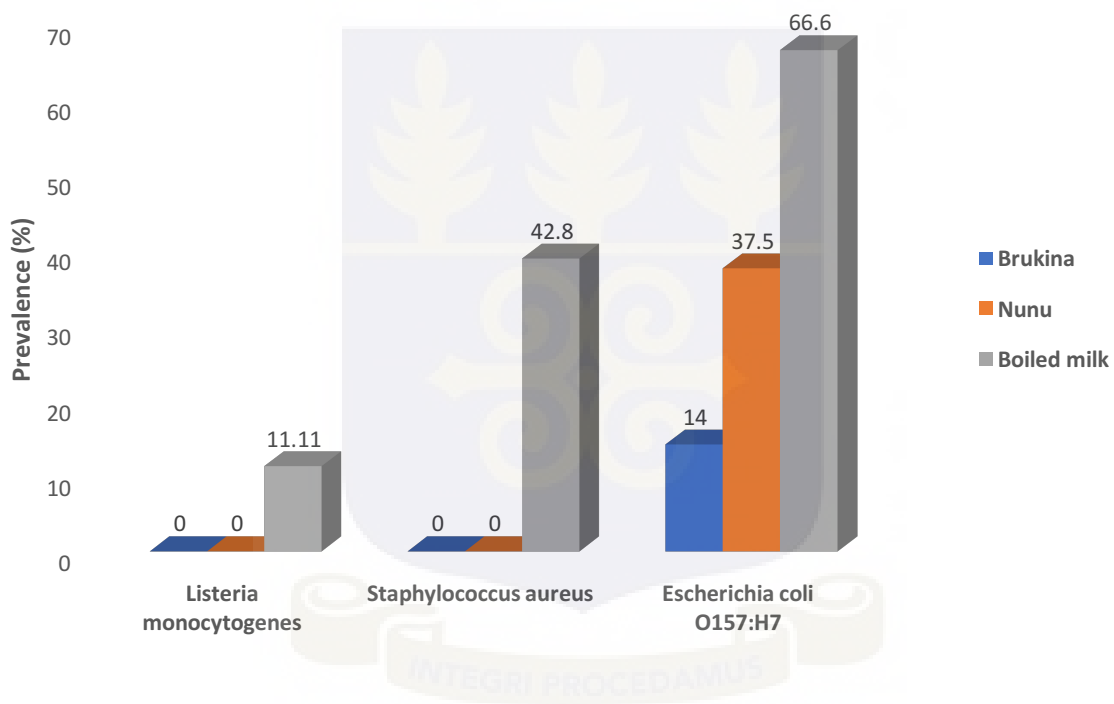


Figure 4: Prevalence of *E. coli* O157:H7, *L. monocytogenes* and *S. aureus* based on qPCR

E. coli O157:H7 was found to be the most prevalent (66.6%) in all the products tested followed by *S. aureus* (42.8%), which was only prevalent in boiled milk and not in *brukina* and *nunu*. However, *L. monocytogenes* was only found in boiled milk, with a significantly low prevalence (11%). The specific detection of *E. coli* O157:H7 in RTE dairy products indicates a contamination of a fecal

origin and ingestion of contaminated food products can cause food-borne illnesses including bloody diarrhea.

Unlike the cultural methods, the molecular analysis showed a higher prevalence of *E. coli* O157:H7 in boiled milk. It is possible that the boiling of milk adequately reduced the survival of the organism to reduce its detection. This is because the molecular analysis could detect the nucleic acid material of the dead cells in the milk.

S. aureus was also detected in the RTE products analyzed. The prevalence indicated by the molecular work corresponds with the findings from the culture work on the samples. Boiled milk was the only sample implicated in the *S. aureus* contamination. This places a major concern on the handling activities involved in the sale of boiled milk. This is because the enterotoxin produced by this pathogen has a high resistance and thus can survive in the food matrix even after the pathogen itself is destroyed.

4.3 Viability of Pathogens in RTE fresh milk products

The specific detection of target genes corresponding to pathogenic microorganisms in an already processed RTE dairy product serves as a significant hazard; however, the extent of the risk to the consumer is dependent upon the state of the target pathogen in the food matrix. All the 18 samples implicated by the results from the qPCR to contain *E. coli* O157:H7, showed growth on the SMAC agar plates. Figure 16 displays *E. coli* O157:H7 isolates growing on SMAC agar plates.

□

E. coli O157:H7

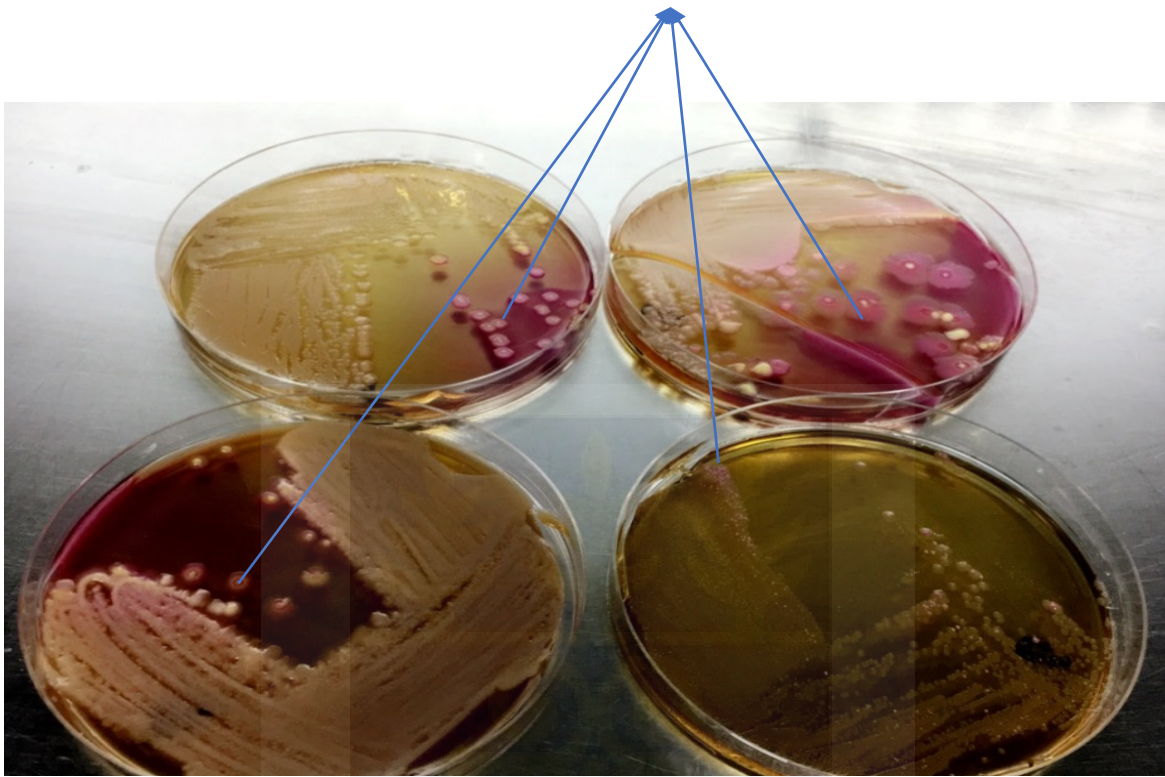


Figure 5: Positive colonies of *E. coli* O157:H7 on Sorbitol MacConkey Agar plates

According to Ceuppens (2014), this indicates a live and infectious state of the pathogen. Ingestion of the pathogen in this state can cause serious health implications to consumers including bloody diarrhea and hemolytic uremic syndrome (Garbaj *et al.*, 2016). Considering that the products subjected to further analysis were RTE fresh milk products, the presence of live and infectious stage of *E. coli* O157:H7 is a clear reflection of inadequate heat treatment or post contamination in the case of the boiled milk and *brukina*.

All the 7 boiled milk samples that tested positive for *S. aureus*, displayed viable cultures upon plating on Baird Parker agar indicating the presence of a live and infectious pathogen. The survival of the pathogen in a heat-treated milk product again indicates a possible defect arising from in the

heat processing or the occurrence of post processing contamination. Ingestion of such contaminated products can cause serious health problems for consumers including staphylococcal food poisoning.

L. monocytogenes was detected in two (2) boiled milk products. The amplification results from the qPCR indicated low level of target genes in the product implicated. This can further be translated into a relatively low level of risk of listeriosis to consumers. This was encouraging, especially considering the comparatively high prevalence of indicator microorganisms such as *E. coli* and *Staphylococcus spp.* However, considering the ability of *L. monocytogenes* to multiply under both cold storage and ambient temperatures, it is noteworthy to consider that beginning low numbers of pathogen can multiply under favorable storage conditions.

The blackening is an indication of hydrolyses of aesculin which could also be mimicked by diptheroids, aerobic spore forming bacilli or *Streptococci* (Kornackiet al., 1993). Upon plating the two products that tested positive for the pathogen, only one out of the two products exhibited viability status.

Figure 17 is a depiction of *L. monocytogenes* isolates growing on Oxford (left) and Modified Oxford agar (right).

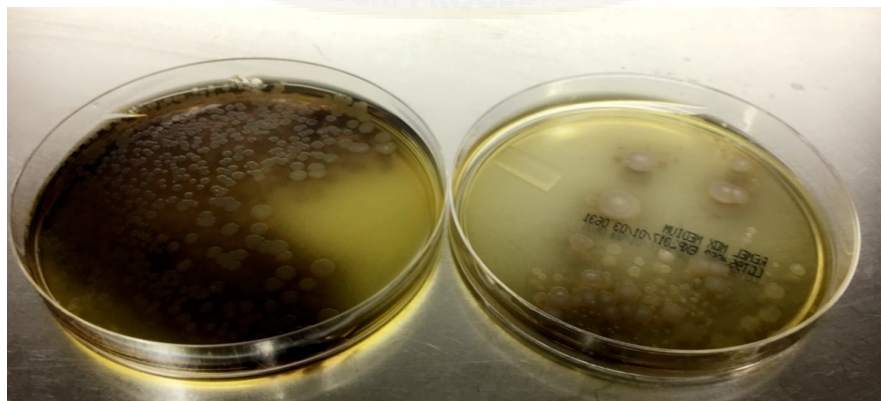


Figure 6: Growth on selective *Listeria* plates

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusions

The analysis of results of fresh milk and fresh milk products suggests a poor microbiological and hygienic quality of the products as evidenced by the concentrations of coliforms, fecal coliforms, *E. coli* and staphylococci in the products tested. The study observed a high incidence of *E. coli* O157:H7 and *S. aureus* in the processed fresh milk product. This indicates unhygienic handling and shortfalls in processing of fresh milk product. This serves as a prompt for food regulatory bodies to increase awareness about the safety of fresh milk products sold in the informal markets.

5.2. Recommendations

There is the need for stakeholders in the dairy value chain to be educated through routine training sessions on effective handling and storage of the fresh milk products to minimize contamination. Again, dairy processors should be encouraged to adopt the usage of starter cultures rather than back slopping. It is recommended that Government agencies and respective food regulatory bodies embark on routine sanitation inspection of market and processing sites and suggest relevant intervention measures for both stake holders along the milk value chain as well as consumers such as good hygiene practices, training, awareness creation and consumer information on milk handling and consumption.

Further investigation should be carried out to characterize the specific strains of *E. coli* O157:H7 detected in this study and to identify the organisms responsible for the false positive blackening of the Fraser broth. This information will be essential to assess the true risk to the consumers

with regards to the milk products marketed informally in Ghana. Further studies should also be done to optimize the heat treatment of milk sold in the informal sector to make sure that the process is indeed a true-kill step in the processing of dairy and dairy products in Ghana

Also, both culture dependent and independent procedures should be encouraged in milk quality assessment to facilitate accurate estimation of the type and state of microorganisms in the milk product.



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APPENDICES

Appendix 1. Analyses of variance

One-way ANOVA: Hygiene and quality versus fresh milk products

	Product	N	Mean	Standard deviation
Total Coliform	Brukina	22	4.420128	2.8466753
	Boiled milk	18	3.612761	3.0650547
	Nunu	8	2.849788	3.0933352
	Raw milk	15	3.641364	2.5645575
	Fried Wagashie	7	.400000	1.0583005
	Raw wagashie	23	5.903913	3.7874623
	Yoghurt	24	1.181250	1.8852293
	Total	117	4.020977	3.2677347
Faecal Coliform	Brukina	22	2.657320	2.0528495
	Boiled milk	18	2.477142	2.3568837
	Nunu	8	2.109901	2.2830777
	Raw milk	15	2.607727	1.8589524
	Fried Wagashie	7	.000000	.0000000
	Raw wagashie	23	4.080435	2.8043335
	Yoghurt	24	0.345000	0.937452
	Total	117	2.546761	2.4394123
E coli Counts	Brukina	22	2.161123	2.0100519
	Boiled milk	18	1.408246	2.0979848
	Nunu	8	1.951374	2.1085543
	Raw milk	15	2.107273	2.1208515
	Fried Wagashie	7	.000000	.0000000
	Raw wagashie	23	2.614783	2.6514042
	Yoghurt	24	2.313365	2.3957314
	Total	117	1.695958	2.1356849

Staph count	Brukina	22	2.746530	1.2507035
	Boiled milk	18	1.936757	2.2502445
	Nunu	8	1.857574	2.1093062
	Raw milk	15	3.098636	1.0866300
	Fried Wagashie	7	.000000	.0000000
	Raw wagashie	23	3.086957	1.7073676
	Yoghurt	24	0.971083	1.3891144
	Total	117	2.249633	1.7692375

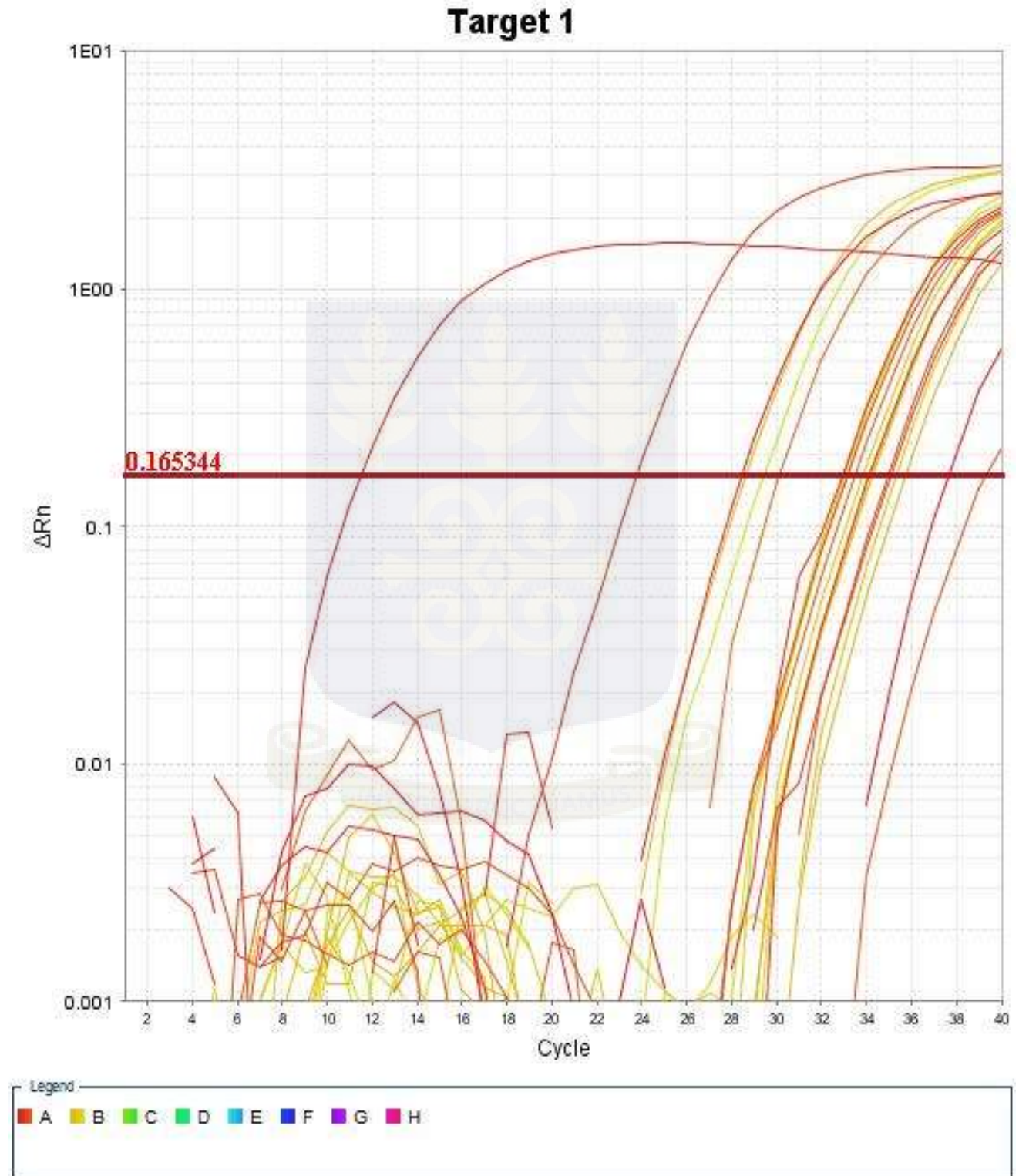


Appendix 2. CT values for target genes *uid*, *hly* and *nuc* for the specific detection of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Staphylococcus aureus* respectively.

Sample ID	<i>uid</i>	<i>hly</i>	<i>nuc</i>
M-1	28.45	UND	37.26
M-2	34.14	UND	UND
M-3	32.92	UND	36.22
M-4	23.81	UND	26.72
M-5	35.07	UND	UND
M-6	33.15	UND	28.41
M-7	34.95	UND	UND
M-8	39.29	UND	UND
M-9	33.51	UND	35.94
M-10	30.12	UND	UND
M-11	35.74	UND	UND
M-12	28.66	UND	34.01
M-13	34.29	37.73	UND
M-14	35.31	UND	UND
M-15	33.86	UND	34.50
M-16	32.99	UND	35.74
M-17	UND	UND	UND
M-18	UND	UND	UND
M-19	34.21	UND	UND
M-20	33.14	37.41	37.07
M-21	29.46	UND	UND

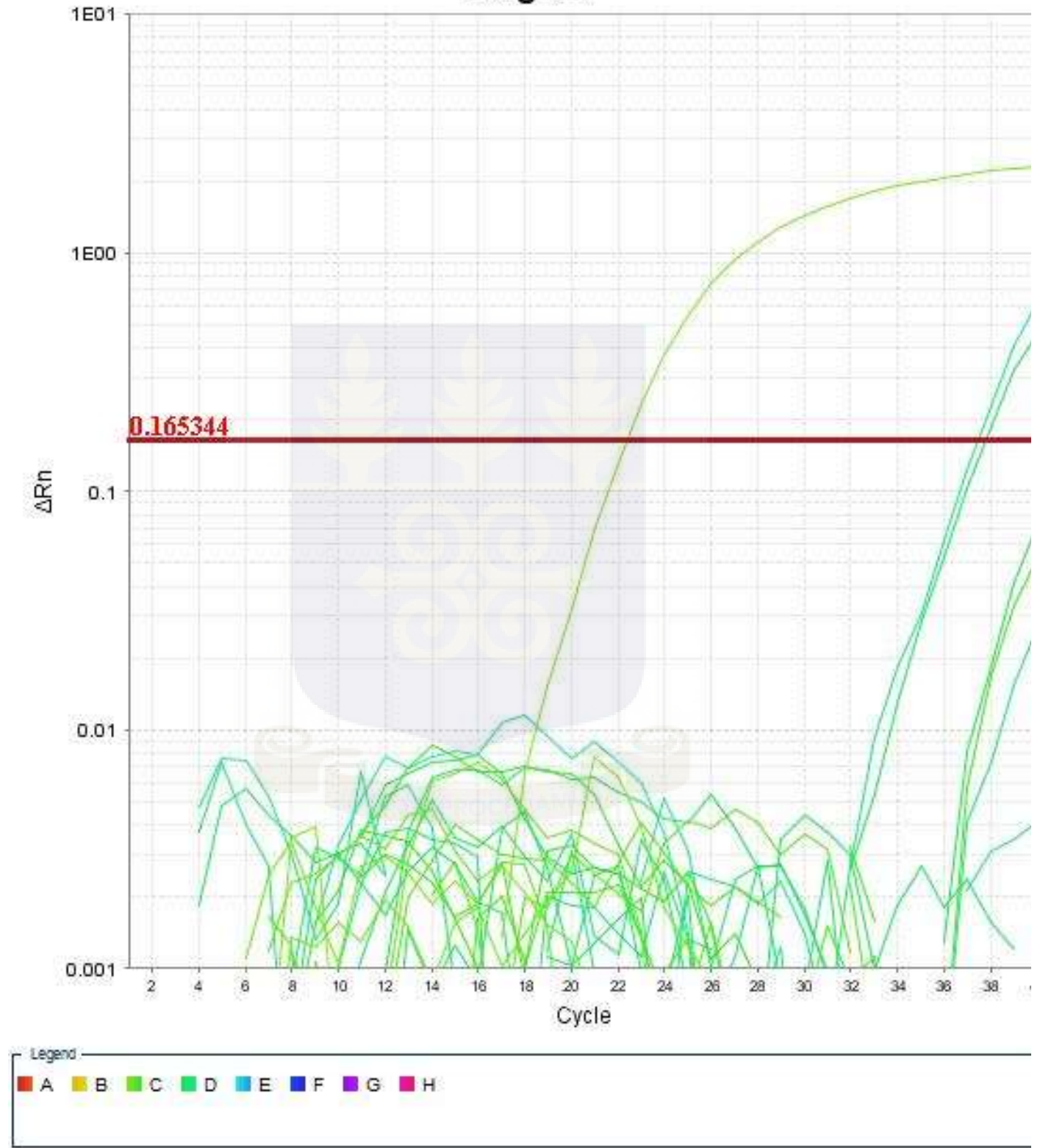
Appendix 3 Real time PCR

Real time PCR amplification plot (ΔRn vs. Cycle) for *Escherichia coli* O157:H7



Appendix 3b. Real time PCR amplification plot (ΔR_n vs. Cycle)for *Listeria monocytogenes*

Target 1



Appendix 3c. Real time PCR amplification plot (ΔRn vs. Cycle) for *Staphylococcus aureus*

Target 1

