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PREVALENCE AND CHARACTERIZATION OF PATHOGENIC *ESCHERICHIA COLI* IN SELECTED INDIGENOUS DAIRY PRODUCT

BY

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THIS DISSERTATION IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MASTERS OF PHILOSOPHY DEGREE.

DECLARATION

I, Dinah Adomako hereby declare that apart from specific references which have been duly acknowledged, this work came out from research undertaken by me at the University of Ghana, in the microbiology laboratory of the Department of Nutrition and Food Science under the supervision of Dr. Angela Parry-Hanson Kunadu and Prof. Kwaku Tano-Debrah.

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DEDICATION

This thesis is dedicated to the Almighty God and for The Work.

ACKNOWLEDGMENT

I thank the Lord for His unfailing love towards me. He helped me through thick and thin and brought me this far. I thank you Lord for putting me in your Work, I do not know where I would have had the chance to get closer to you. ‘THANK YOU, LORD,’

I also want to thank all the saints who supported me in my school most especially Brother Zac Shadow, I am most grateful, the Lord bless you abundantly.

I am grateful to my family for their encouragement and their help in all my education. I cannot say enough, thank you, uncles.

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ABSTRACT

Diarrhoeagenic *E. coli* is a major health concern. It causes mortality and morbidity especially in children of age five years and below in countries that are developing. Research has revealed that out of the six categories of diarrhoeagenic *E. coli*, Enterohaemorragic *E. coli* (EHEC) and Enteropathogenic *E. coli* (EPEC) are the most widespread cause of diarrhea. This work identified the existence of some *E. coli* that causes diarrhea in informal dairy products from selected traditional open markets and streets of Greater Accra and characterized the survival of *E. coli* O157:H7 in Mueller Hinton broth and *wagashi*, a soft unripened indigenous cheese. A total of 85 local dairy products, specifically raw milk, boiled milk, *brukina, nunu* and *wagashi* were examined. These were purchased from cow rearing locations and markets within Accra. *E. coli* was detected by enrichment in Brain Heart Infusion broth followed by plating on MacConkey agar. *E. coli* O157:H7 was determined on Sorbitol MacConkey agar which appeared colorless. Survival of *E. coli* O157:H7 to sub-lethal stresses of low pH, high salt and heat was determined in broth and at multiple processing stages of traditional *wagashi*. Out of the 85 milk samples, 60 (70.6%) tested positive for *E. coli*. Prevalence of 6 categories of diarrhoeagenic *E. coli* was determined by Multiplex Polymerase Chain Reaction (mPCR) using standard primers for their virulence genes. The multiplex RT PCR assay also detected 45 samples that tested positive for diarrheaogenic *E. coli*. Out of the 45 positive samples, 14 samples (26.7 %) were positive for Enterohemorrhagic *E. coli* (EHEC), 3 (6.7 %) were positive for Enteroinvasive *E. coli* (EIEC), 2 (4.4 %) were positive for Enteropathogenic *E. coli* (EPEC), 3 (6.7 %) were positive for Enterotoxigenic *E. coli* (ETEC) and 4 (8.9 %) were positive for Enteroagreggasive *E. coli* (EAEC). The remaining 19 (42.2 %) samples

contained a mixture of two or more diarrheaogenic *E. coli* virulence genes. The presence of diarrhoeagenic *E. coli* in informal diary suggests that the consumption of the informal dairy products tested in this study could be a public health risk. Since *E. coli* is an indicator of poor hygiene in foods the study recommends food hygiene training for stakeholders and development of hygienic standard operating procedures for use by all stakeholders in the informal milk value chain to prevent or reduce consumer exposure to diarrhoeagenic *E. coli* through consumption of informal dairy products.

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CHAPTER 1

1.0 INTRODUCTION

1.1 Background

Milk is known as an extraordinary nutritional food (Herrmann and Carroll, 2014). It is popular for its delicious taste and vital nutrients, like protein, calcium, potassium, phosphorus, fats, vitamin A, B1, B2, B12, riboflavin and niacin (Castro, 2017; Hoddinott *et al*., 2015). The high source of calcium in dairy products is very vital for bone formation in infants as well as significantly decreasing the risk of osteoporosis-induced fractures mostly encountered by the elderly after 50 years of age (Kim *et al*., 2015; Rovira, 2015; Valverde and Gómez, 2015).

However, dairy is an exceptional habitat for microbial development and an efficient vehicle for transmission of enteropathogens to humans because of its rich water content and complex biochemical constituent (Amenu *et al*., 2014; Pappa *et al*., 2017). About 90% of food borne diseases caused by pathogenic bacteria are connected to ingestion of milk products. Studies on characterization of microorganisms from products that contain substantial amount of dairy have been reported by such as Iran (Bonyadian *et al*., 2014), (Kumar and Kannan, 2018), (Dadie *et al*., 2010), (Paneto *et al*., 2007), (Ivbade *et al*., 2014), (Hessain *et al*., 2015) in various countries. According to Luna-Gierke *et al.* (2014) and Heiman *et al*. (2015), one of the most predominant food borne pathogen recorded in milk is *Escherichia coli*.

E. coli is an organism that is gram negative and mostly inhabits the intestinal tract of humans and warm-blooded animals. Some strains of *E. coli* do not cause are harm and beneficial as they help in the breakdown of food in the intestine. In view of this, *E. coli* was considered to be harmless in the past and its presence in food was only an indicator of poor hygiene and a reliable indicator of fecal pollution (Olalekan *et al.,* 2019). However, several *E. coli* groups are tagged with pathogenic significance as they cause food borne illnesses. Some group of strains are harmful as they poison foods that humans ingest. *E. coli* O157:H7 is one of the food pathogens that has emerged in the dairy industry that presents significant risks to public health (Cortés-Sánchez and Salgado-Cruz, 2017). Cattle, which has been established as an asymptomatic host of Shiga toxin-producing *E. coli* (STEC), may spread diarrheagenic *E. coli* to humans through human consumption of STEC contaminated meat, milk or by direct contact with cattle or its fecal matter (Haiqing *et al.,* 2016). Diarrhoeagenic *E. coli* has been identified through research from cheese and dairy products in countries like, Bangladesh (Tanzin *et al*., 2016), and India (Kumar and Kannan, 2018). These microorganisms can infect individuals who are either healthy or immunocompromised, including pregnant women, older adults and unborn fetuses. Illnesses as a result of infection with diarrhoeagenic *E. coli* range from diarrhea to life-threatening fetal miscarriage and kidney failure (American Academy of Pediatrics, 2014).

In Bangladesh, approximately 20 % of all diarrheal cases recorded in children are Enterotoxigenic *E. coli* related which is also a predominant strain (Tanzin *et al*., 2016). According to the American Academy of Pediatrics (2014), 82 % of milk products associated to foodborne disease outbreaks reported by the United States Center for Disease

Control and Prevention (US CDC) from 1973 to 2009, were attributed to the consumption of raw milk or cheese.

Regulatory bodies have come up with guidelines on hygienic handling and processing of milk products, nevertheless, there are numerous challenges with adoption of the guidelines especially in the developing countries thereby increasing the risk of milk-borne diseases (American Academy of Pediatrics, 2014; Shaker and Elaref, 2015).

Problem statement

In Ghana dairy production and consumption is low, whiles economic growth of the fresh dairy business, predominantly in the informal sector, is stagnant (Karamba *et al*., 2011). Locally produced cow milk is typically processed into traditional fermented foods where compliance to standardized pasteurization and hygienic processing operations is minimal. The consumption of informal dairy products is gradually increasing due to increasing income levels and availability in most street corners and in the market places throughout Ghana. Conversely, public health concerns related to consumption of these milk products are increasing, possibly due to the unhygienic handling of these locally produced products (Kim *et al*., 2015; Parry-Hanson Kunadu *et al*., 2019). Most traditional dairy products sold in Ghana are unregulated therefore safety is not monitored.

Owusu-Kwarteng and Wuni (2018) reported on the microbiological quality of the milk value chain in Ghana. Owusu-Kwarteng *et al.*, 2017 and Parry-Hanson Kunadu *et al.* (2018) also characterized dominant microorganisms in traditional dairy products. However, to the knowledge of the author, reported study on diarrhoeagenic *E. coli* characterization in informal dairy products in Ghana is not available. This work therefore

is intended to provide information on the prevalence of diarrhoeagenic *E. coli* in traditional Ghanaian dairy product as well as characterize the survival of selected *E. coli* O157:H7 strains to *wagashi* processing treatments.

1.2 Rationale

E. coli is one of the frequently reported microorganisms identified in milk (Tankoano *et al*., 2016). Statistics from Ghana's Ministry of Health indicate that diarrhea accounts for 84,000 deaths annually with 25 % being children under five years (Ghana News Agency, 2003). Diarrhoeagenic pathogens go a long way to cause damage in the intestinal epithelium and its absorption function reduces resulting in the depletion of nutrients (Pawłowska and Sobieszczańska, 2017). In Ghana, *E. coli* among many fecal pathogenic organisms such as *Streptococcus spp., Klebsiella pneumoniea* have an average prevalence rate of 70 % in traditional dairy products in Accra (Parry-Hanson Kunadu *et al*., 2019). Thus, dairy products signify a significant source of food-borne pathogens. These pathogens that colonize dairy products have been linked to improper practices in the dairy chain from the farm-gate through to the consumer. In Ghana, year-round milk production and processing are mostly the occupation of the nomadic Fulani families who put up temporary structures to live in when they find good pasture for their cattle. Such structures do not comply with hygienic design thus conditions of production and processing of milk exposes the products to microbiological contamination. It was reported in a recent study that poor sanitation and unhygienic practices in the course of milking and processing of local milk products by producers and vendors resulted in poor patronage of the dairy products (Parry-Hanson Kunadu *et al.,* 2019). Specifically, the lack of effective hygienic practices such as the use of unsanitary equipment for milking, lack of proper handling of the dairy products,

lack of proper appropriate cold chain facilities and poor transport systems affect the suitability and safety of dairy products. Such lack observed from the farm-gate to the consumer puts the health of consumer at risk.

However, the enormous nutritious benefits and the high energy content in dairy products is invigorating the demand of local milk products especially *brukina* on the streets of Greater Accra and the interest in promoting fresh milk consumption (Jerry *et al*., 2020). It is therefore essential to minimize contamination during production. It is also crucial to assess the storage quality by assessing the growth of diarrhoeagenic *E. coli* under some lethal processing conditions after adaptation. Studies have revealed adaptive systems in *E. coli* which when activated confer cross-protection to other lethal processiing and storage conditions. These adaptive systems are employed by some pathogenic strains of *E. coli* such as the diarrhoeagenic *E. coli* in order to survive in unfavorable processing conditions (Hsin-Yi and Chou, 2001).

Assessment of risk of foodborne disease depends on knowledge of hazard characterization in food products. While some prevalence/concentration data on *E. coli* O157:H7 are available, there is limited/no data on other diarrhoeagenic *E. coli* and their resistance to processing technologies for local dairy products. This work therefore intended to provide information on the prevalence of diarrhoeagenic *E. coli* in traditional Ghanaian dairy products and also assess the survival of a selected *E. coli* O157:H7 strain in *wagashi* processing treatments.

1.3 Objectives

1.3.1. Main objectives

To determine prevalence of diarrhoeagenic *E. coli* in informal dairy products and investigate responses relating *E. coli* O157:H7 that has been acid-adapted to treatments unusual in processing in *wagashi*, a traditional Ghanaian dairy product.

1.3.2 Specific objectives

1. To determine the prevalence of diarrhoeagenic *E. coli* in informal dairy products.

2. To determine phenotypic responses of acid-adapted *E. coli* O157:H7 to low pH, sublethal heat, and salt stresses in broth.

3. To assess the survival and growth potential of acid-adapted *E. coli* O157:H7 during *wagashi* processing treatments and abuse storage condition.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Dairy source and production

Dairy are foods produced as a result of processing milk or foods containing significant quantities of milk. Milk production is currently dominated by five animal species namely cattle, goat, sheep, buffalo and camels (Barlowska *et al.*, 2011).

Milk is a liquid suspension secreted from the mammary glands of mammals that is used to feed their young ones. They therefore contain energy and nutrients needed for the growth of the newborn mammal. The exact nutritional composition varies from species to species. Throughout the history of man, cows have played a role in the welfare of man by supplying milk for consumption (Appiah, 2012; Gemechu *et al.*, 2014; A. Parry-Hanson *et al.*, 2009c)

Dairy products are rich in both saturated and unsaturated fat, proteins, essential minerals (potassium, magnesium, calcium and zinc), vitamins A, B, D, E and lactose. The chemical composition of milk differs with respect to different animal sources from which it is obtained. Table 1 indicates the chemical composition of milk derived from common dairy animals.

Research evidence continues to enlarge the beneficial role of milk and milk products on immunity, reduction of selected cancer ailments, mild hypertension, supporting weight management strategies, increasing satiety in dieters (Hess *et al.*, 2016) and reduced risk of infection with non-transmissible chronic diseases (Reale *et al*., 2015). Furthermore, an

inverse correlation has been reported between the consumption of dairy products and colorectal cancer, stroke and hypertension (Álvarez-Ordóñez *et al.,* 2013).

Table 1: Chemical composition of milk from the five main animal sources

Species	Protein%	Fat%	Lactose%	Reference
Cow	3.42	4.09	4.82	Auldist *et al*., 2004
Sheep	5.73	6.99	4.75	Ahmad *et al.*, 2008
Goat	3.26	4.07	4.51	Sanz *et al.*, 2009
Camel	3.26	3.80	4.30	Mahmuda *et al*., 2014
Buffalo	4.38	7.73	4.79	Tripaldi *et al*., 2010

Milk consumed in Ghana is predominantly from cattle which are extracted using crude methods by herdsmen who are mostly Fulani men. Fulani men travel from Nigeria, Mali, Niger, Chad and other northern African countries. Their main occupation in Ghana is to look after herds belonging to wealthy individuals with numbers ranging from a minimum of 10 cattle. They are remunerated from proceeds of milk sold and also young female calves to enable them start their own herds (Gidiglo, 2014). Cattle in Ghana provide food (meat and milk), manure and manpower for farming in certain towns and villages. Data from FAOSTAT, (2018) estimates domestic milk production at 38,000 tonnes per annum which together with the meat represents about 30% of the protein requirement of the country. This puts the consumption of milk at less than 120kg per annum which is the required minimum (Gidiglo, 2014).

The report of the (FAOSTAT, 2010) indicates that the world milk production accounted for a total of 696.6 million kg^3 of milk out of which 580.5 million kg^3 (83.3 %) was cow milk, 90.3 million kg^3 (13 %) buffalo milk, 15.1 million kg^3 (2.2 %) goat milk, 9 million kg^3 (1.3 %) sheep milk, and 1.6 million kg^3 (0.2 %) camel milk (Barlowska *et al*., 2011).

In 2002, milk production in Ghana alone was estimated at 34,000 metric tonnes (Addo *et al*., 2011). This was supported by annually importing an average of 37,195 metric tonnes of liquid milk equivalent (LME) to make up the deficit (Addo *et al*., 2011). Consumption of pasteurized raw milk in Ghana is uncommon as most people consume condensed full cream evaporated milk and powdered milk. The reason being that production of milk in Ghana is very low causing low patronage and consumption as well. Okantah *et al.*, (2006) reported that $39{,}831.4\times10^3$ tonnes of LME were imported into the country from 1995 to 1999.

According to FAOSTAT, (2018) in table 2, between 2007 and 2017, there was an average production of 1,561,544 cattle out of which only 300,587.8 cattle on the average were for the production of milk. Milk producing cattle constituted on the average 19.25 % of the total cattle production in Ghana.

Table 2: Cattle production in Ghana

YEAR	TOTAL CATTLE	MILK PRODUCING CATTLE	PERCENTAGE OF MILK PRODUCING CATTLE
2007	1,373,000	285,500	20.79%
2008	1,392,000	286,450	20.59%
2009	1,438,000	289,700	20.15%
2010	1,454,000	269,375	18.53%
2011	1,498,000	300,000	20.03%
2012	1,543,000	315,000	20.42%
2013	1,590,000	315,000	19.81%
2014	1,657,000	301,231	18.18%
2015	1,734,000	313,134	18.06%
2016	1,734,000	313,207	18.06%
2017	1,763,984	317,869	18.02%
AVERAGE	1,561,544	300,587.8	19.25%

Data Source: FAOSTAT, (2018)

In 2013, 40,950 tonnes of whole milk was produced in the country in an effort to meet the demand for milk by consumers. To augment this, high volumes of milk are imported. There is an average importation of 44,347 tonnes of whole milk and 108,195 tonnes of skimmed milk annually to meet the demand of milk in the country.

Based on the high demand of milk for consumption and other dairy products, FAO (2012) has encouraged commercial cattle farmers to focus on the production of milk to serve consumers and this comes at the back of the low yields recorded from the low numbers of milk producing cattle as shown in tables 3, 4 and 5 below.

Table 3: Whole milk Source in Ghana (Tonnes)

YEAR	Produced	Domestic supply	Quantity imported
2003	35,100	49,033	16,503
2004	35,490	69,683	41,044
2005	36,010	61,500	48,884
2006	36,530	84,708	50,668
2007	37,120	79,670	54,857
2008	37,238	83,631	59,051
2009	37,661	62,620	28,456
2010	38,701	77,836	42,414
2011	39,000	89,567	57,693
2012	40,950	73,585	38,064
2013	40,950	84,234	50,193
AVERAGE	37,704.55	74,187.91	44,347.91

Data Source: FAOSTAT, (2018)

Table 4: Skimmed milk and cheese (Tonnes)

YEAR	Domestic supply		Quantity exported		Quantity imported	
	Skimmed milk	Cheese	Skimmed milk	Cheese	Skimmed milk	Cheese
2003	80,277	218	988	0	81,265	218
2004	92,909	253	1,382	10	94,291	263
2005	90,784	312	4,833	0	95,617	312
2006	106,559	315	803	1	107,362	316
2007	105,148	405	161	34	105,309	439
2008	130,945	419	600	21	131,545	440
2009	74,487	387	20	10	74,507	396
2010	106,449	425	40	0	106,489	425
2011	134,150	635	40	0	134,190	635
2012	118,791	653	542	2	119,333	655
2013	140,212	656	30	0	140,242	656
AVERAGE	107,337.4	425.27	858.09	7.09	108,195.5	432.27

Data Source: FAOSTAT 2018

The low yields of the cattle are due to genetic and non-genetic factors. Averagely, cattle in the tropics have lower milk yields and shorter lactations than cattle in the temperate (Syrstad, 2018).

Table 5: Milk producing cattle and their yields in Ghana

YEAR	TOTAL CATTLE (TONNES)	YIELD (Hectogram per Animal) Hg/An
2007	37,120	1,300
2008	39,804	1,390
2009	40,179	1,387
2010	37,865	1,406
2011	41,356	1,379
2012	43,047	1,367
2013	43,053	1,367
2014	41,515	1,378
2015	42,858	1,369
2016	42,873	1,369
2017	43,401	1,365
AVERAGE	41,188.27	1,370.636

Data Source: FAOSTAT 2018

Most of the cattle farmers do not have enough cows and therefore keep them in a kraal. Milking of the cows are done in this same kraal. When the animals are ready to be milked, they are isolated and milked each day before they are taken out for grazing. The milking is done by the herdsmen with support from their families as shown in fig. 1, but in the case of larger farms, laborers are hired to assist with the process. In most cases during milking, the legs and tail of the cows are tied to pillars or trees to prevent tail wagging and moving of legs which results in contamination of the milk. The milk is collected in cooking utensils and buckets for storage and processing as described by Akabanda *et al*., 2010.

Figure 1: Milking of cow by a Fulani woman (Source: Akabanda *et al*., 2010)

The utensils used are often not stored under clean hygienic conditions. Due to the location of most of these cattle farms they lack clean potable water and therefore use water from wells and dams to wash their hands and utensils before the milking begins.

Because of the size of the farms, milk harvested from the cows are averagely 13.5litres daily (Gidiglo, 2014) These low volumes are not enough to be processed on a large scale to meet customer demands. There are people who bridge the gap by working as aggregators. These aggregators often move from farm gate to farm gate buying the smaller volumes of the milk and sell them to wholesalers. These wholesalers then sell the accumulated milk from the aggregators to processors who use them for *wagashi*, fresh yoghurt, *brukina*, hard cheese and *fura* as observed by (Gidiglo, 2014). This produces a long chain of handlers in the process as shown in fig. 2, therefore, exposing the milk to possible contamination.

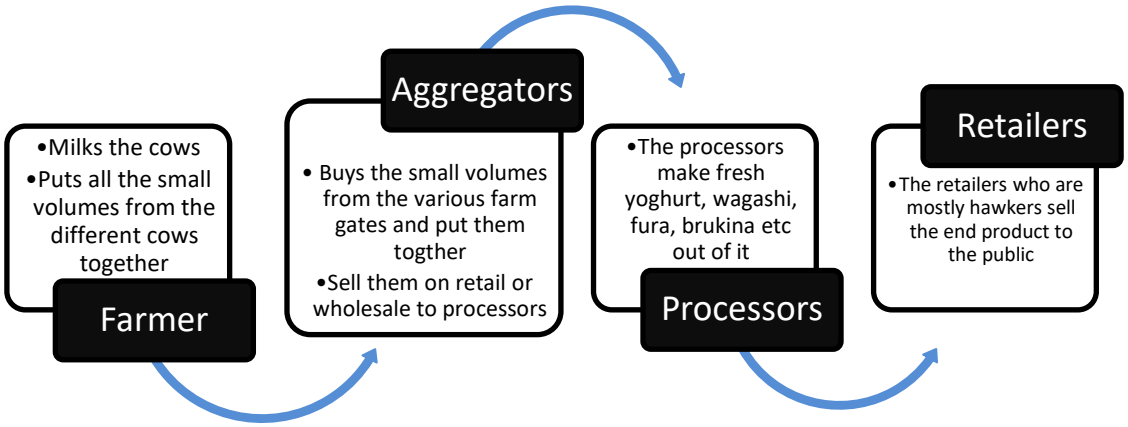


Figure 2: Process flow of diary production and sale in Ghana

Though Ghana has a high number of animals that produce milk, the annual consumption per individual is lesser than the required minimum by the Food and Agriculture Organization. This can be attributed to the high poverty rates in the country and the relative high cost of milk production. Milk in its unprocessed form is perishable and very bulky to transport adding to the cost of production. Other difficulties the dairy industry encounters in Ghana includes increased number of low milk producing cattle as most of the cattle are reared for their meat and not their milk, non-compliance to good livestock farming practices made up of absence of vaccinations, and veterinary services also adds up to the already existing challenges. This does not apply to only Ghana but other African countries as well. Ngigi, (2003) found that as much as 80 % of the milk supplied in rural and semi-urban areas in Kenya is produced, processed and marketed in the informal market without adhering to standards and best practices. This lack of standards and proper handling of milk locally is one of the major reasons Ghanaians prefer imported milk to the locally produced

ones. Parry-Hanson Kunadu *et al*., (2019), supports this assertion by indicating that the perception that milk produced locally are made under unhygienic conditions and unsafe makes Ghanaians prefer imported milk.

For production of cattle to rise and other livestock in the country, the Ministry of Agriculture has established the Livestock Breed Improvement program (LBIP) to supply improved breeds of livestock to farmers across the country (Ferro *et al*., 2017) . According to the Medium-Term Agriculture Sector Investment Plan (METASIP), between 2002 and 2006, the Agricultural Services Sub-Sector Investment Program (AgSSIP) was established to reinforce and support the rapid agricultural growth and aid in poverty reduction. These programs and policies form part of measures put in place to improve technology and practices of farmers. The specific interventions to exploit the vast opportunities available in the production of cattle and milk includes the rehabilitation and capacity building of breeding stations to improve livestock breeds for farmers (Ministry of Food and Agriculture, 2010). In addition, introducing efficient health interventions and the establishment of sustainable artificial insemination services will help to improve the fortunes of the cattle farmers and herdsmen in achieving maximum productivity levels. The programs also promoted communal grazing lands for cattle farmers in addition to the support provided for the establishment of pastures.

Sintayehu *et al.* (2008) points out that to be able to increase milk production by farmers with smaller number of cattle, there is the need for marketing outlets or ready off takers as was experienced in Uganda and Kenya, because this will serve as a motivating factor. The METASIP therefore facilitates the linkage of producers to marketers and promote value addition to livestock and farm produce (Ministry of Food and Agriculture, 2010).

2.2 Dairy consumption in Ghana

Milk in Ghana is marketed and distributed as fresh milk and also processed into different dairy products. The widely patronized milk products in Ghana consists of evaporated milk, powdered milk, condensed milk, yoghurt, cheese (local and imported) and raw fresh milk. The consumption rankings for the commonly patronized dairy products are presented in table 6. According to Walton, (1998), the behavior patterns of consumers in terms of purchases are greatly influenced by personal, household and the characteristics of the product coupled with economic and other cultural factors.

Table 6: Milk product Type consumed and their consumption rankings in Accra and Kumasi of Ghana

MILK PRODUCT TYPE	**Percentage**	RANK
Evaporated milk	89.12	1
Yoghurt	85.15	2
Powdered Milk	68.98	3
Raw fresh milk	44.55	4
Ice cream	42.90	5
Butter	36.63	6
Wagashi	28.05	7
Fermented Milk	23.10	8
Condensed milk	19.80	9
UHT milk	9.90	10
Boiled milk	7.59	11
Imported cheese	4.29	12

Source: Aidoo *et al.* (2009) Key: 1= the most patronized, 12= the least patronized

Mullins *et al.,* (1994) found that there are three main determinants of dairy consumption and they include ethnic backgrounds, level of household revenue as well as locality of the household. Appiah (2012) also adds that the unreliable assembling systems available and the unappealing costs presented by producers for the raw milk make people turn towards imported skim milk powder and evaporated milk. The source of dairy products in the

formal sector is primarily from the importation of dairy and milk products and a little portion from the domestic market. That notwithstanding, a substantial amount of the milk is collected from the kraals but does not often get to the formal market because they are consumed by the households and families of the herdsmen and the local informal markets.

Ghanaians are yet to develop the habit of regularly drinking milk. Only a small number of people in any particular household consume milk and it applies to children in the household as well (Cortés-sánchez and Salgado-cruz, 2017) (Aidoo *et al*., 2009). Powdered and liquid milk are commonly used as creamers for breakfast beverages and porridges.

2.3 Local Indigenous Dairy products

Dairy is used in several food products to enhance its nutrition, flavor and taste (Eaci *et al*., 2017). Depending on the country and culture, milk is processed into different dairy foods or used as constituent in foods. Milk is mostly consumed as pasteurized liquid milk or used in producing dairy products like sour cream, sour milk yogurt and cheeses in the United States. In Spain some common dairy products are curd, curd snack, caujada and custard. In South America some milk products are Dulce de leche, doogh (a confection prepared by heating sweetened milk popularly 'condensed milk toffee' in Ghana) and Eggnog (also known as milk punch) (De Melo *et al.*, 2014). In Turkey some milk products consumed are kaymak (similar to clotted cream), milk kefir, *kashk* (caramelised yoghurt), *khoa* (similar to ricotta cheese), *kulfi* (Tokuşoğlu *et al.*, 2004). In Africa milk has also been used for, or included in several products by either fermenting or used in its raw state. In Kenya some of the dairy products are *paneer* and *murski* (Renuka *et al.*, 2009) and in Ghana *brukina*, *wagashi* and *nunu* are some dairy products consumed. Apart from Ghana, *wagashi* is a

dairy product common in Niger, Mali, Burkina Faso, Mauritania, Togo, Ivory Coast, Nigeria and Benin.

Other local dairy products include *maishanu cuku* (a cheese made by the Fulanis) and *wara* (a cheese made by the Yorubas).

2.3.1 Traditional milk products in Ghana

In Ghana, milk products are either sold as fresh raw milk or as fermented traditional milk products. There are also fermented cereal-milk products that are indigenous. These milk products are mostly fermented as a way to preserve them for longer days due to the absence of proper modern storage facilities in the farming communities dominated by cattle rearing.

2.3.1.1 *Wagashi*

Wagashi is an indigenous unripen soft cheese produced from cow milk. It is widely consumed in a number of West African countries including Ghana, Benin, Nigeria and Togo. It is mostly produced by wives of Fulani pastoralists who process surplus raw milk from raising of cattle. *Wagashi* (as shown in fig. 3) is produced by mild heating the milk to an average temperature of 55 °C, and coagulated with the extract of Sodom apple plant (*Calotropis procera*) which has an enzyme called calotropain (Zecchini and Cantàfora, 2010). The Sodom apple is washed and crushed with a little amount of water. It is then filtered and added to the heated milk. Extract allows for coagulation of the milk, which is then drained and molded for sale in the informal market as shown in fig. 4. It is fried in oil, smoked over fire or cooked in soups as protein before consuming. It can be stored in a refrigerator or whey to maintain the moisture content and prevent it from going bad. It can

also be dried on a rooftop and can be stored for a maximum of 45 days without any significantly changes to the organoleptic characteristics (Dossou *et al.*, 2006).

Figure 3; *Wagashi* (a dairy product)

Wagashi is one of the major sources of protein and other nutrients in communities with low-income levels. When smoked or fried, they are served as replacement for meat and eaten with rice, banku, kenkey and any other food that hitherto would have been eaten with fish or meat.

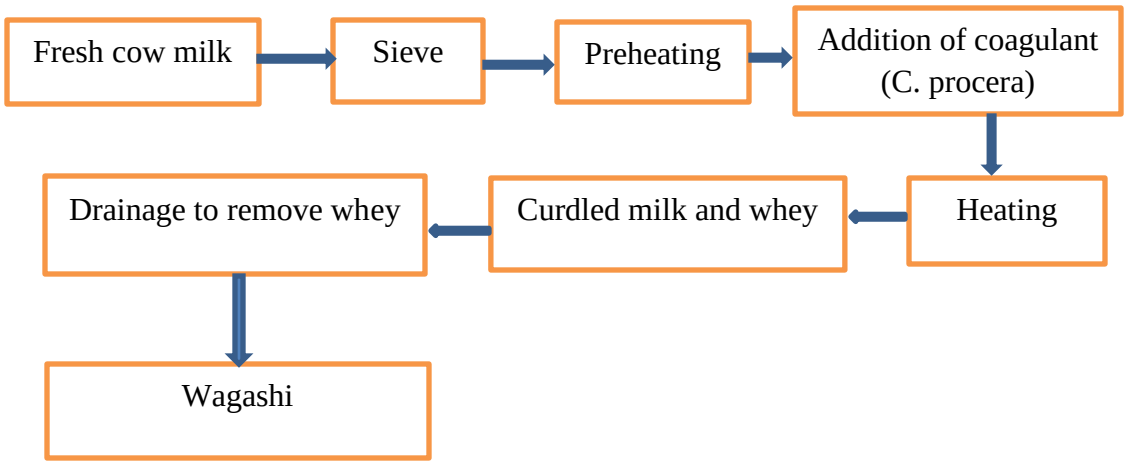


Figure 4: Process flow for preparation of *wagashi*

2.3.1.1.1 Preservation of wagashi

Generally, *wagashi* is stored at room temperature (28℃) in its whey. In this condition, the shelf-life of *wagashi* is 2 or 3 days since it is highly perishable (Belewu *et al.*, 2005). To prolong the shelf life, processors brine the whey in which *wagashi* is stored. Storage in high concentration of salt has been shown to lengthen the shelf life of *wagashi* reaching about 20 days at room temperature (Appiah, 2012). In addition to extending the usable shelf-life of *wagashi*, salt also influences its flavor and texture. Coker *et al*., (2005) explained that salting is done by either adding directly to the *wagashi* after running off the whey, i.e. before pressing the curd into molds or by submerging the formed curd in brine for about 2-3days. This reduces the water activity (moisture content) of the *wagashi* thereby not making it habitable for microbes.

The level and technique for salting impact pH changes in the *wagashi*. Coker *et al*., (2005) added that adding salt to the cut curd draws more whey from the *wagashi* and a portion of the salt diffuses into the curd. Coker *et al.,* (2005) indicated that the pH of the curd, the

contact time and the salt molecule size and structure are essential in deciding how much salt is diffused into the *wagashi.* Salt is additionally associated with physical changes in the protein dissolvability and conformation, which impact the rheology of the product. Another vital role of salt in *wagashi* is as a flavor enhancer (Yoon *et al.*, 2016).

According to Sessou *et al*., (2013), *wagashi* is can be sun dried or heated daily to extend its shelf life to about 12days though smoking makes it last about 30days. These processes directly kill the microbes and/or reduce the water activity of the *wagashi* thereby reducing the spoilage activities of the microbes.

2.3.1.2 *Nunu*

Nunu is a fermented milk product that looks closely like yoghurt (shown in fig. 5). It is very common in most West African countries. It is called by different names depending on the locality and the slight variations in its processing. In the northern parts of Ghana, Burkina Faso and Nigeria it is called *nunu*, *nyarmie* is used in southern Ghana, fènè in Mali and lait caillé in Mali (Obodai and Dodd, 2006; Wullschleger, 2009).

Figure 5; *Nunu* (a diary product)

Nunu is prepared by using fresh cow milk. The milk is sieved to remove foreign materials like fur and other particles that have found their way into the milk during the process of collecting it from the cow. The milk is made to ferment for one or two days in a calabash or plastic containers. The main difference between the production of *nyarmie* and *nunu* is that in the production *nyarmie*, the milk is pasteurized and defatted before fermentation whereas in the production of *nunu* it is absent as shown in fig. 6. This fermented milk is used as a starter culture to inoculate freshly drawn cow milk in some instances for new batches and to allow fermentation at ambient temperature for 24 hours. During fermentation, lactose is converted to lactic acid. The fermentation provides the *nunu* sharp acidic taste. Towards conclusion of maturation period, churning is done by agitating the overnight milk to remove the milk butter to obtain the sour milk called *nunu* (Olalokun, 1996). According to Akabanda *et al.*, (2010), the production of *nunu* in the Northern parts of Ghana does not involve the use of starter culture for inoculation but instead the milk is fermented by microbes arising spontaneously from the environment processing equipment

or processors. Yahuza (2001) adds that because of this unhygienic way of preparing and the short shelf life of *nunu*, it does not appeal to a lot of people.

It can be left without refrigeration for a couple of days without it going bad. *Nunu*, is consumed either as a refreshing beverage with sugar or fura (fermented cereal dough) or can added as an ingredient to foods or used as weaning food. In the northern parts of Ghana and the southern parts of the country that have a huge population of migrants from the northern sector of the country, it is usually in high demand during the Ramadan (a period of fasting for Muslims).

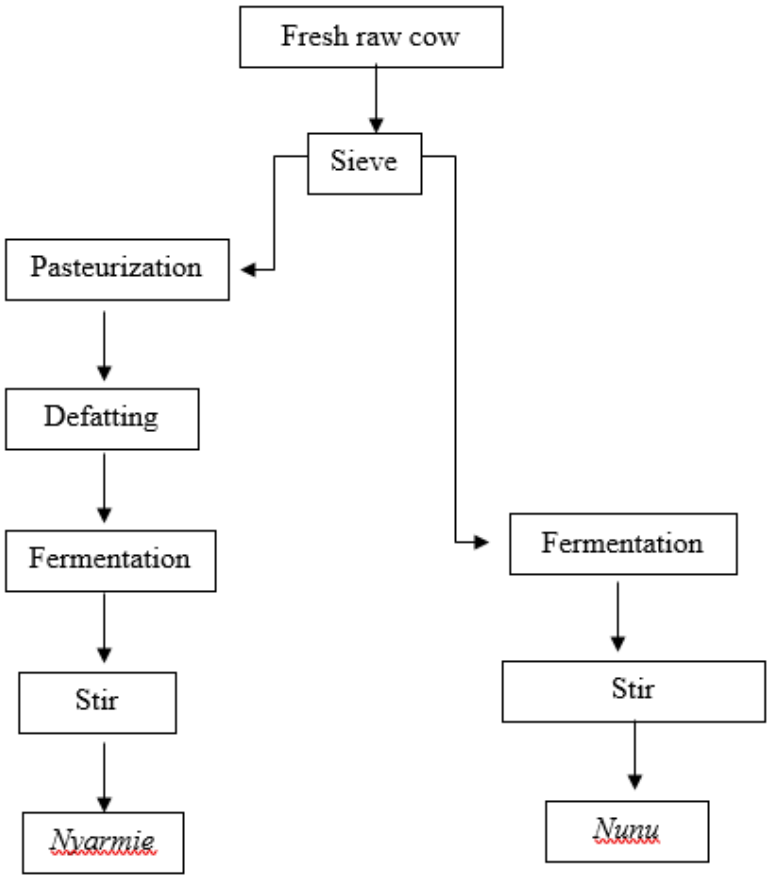


Figure 6: Traditional processing of *Nunu* and *Nyarmie*

Source: (Akabanda *et al*., 2010)

2.3.1.3 Brukina

Brukina is a smoothie made of fermented millet dough and fermented milk which is very popular in most Northern parts of the country and also communities dominated by people from the northern parts of the country. Its origin is Burkina Faso- a country Ghana shares its northern border with. Its production is produced on a small scale and dominated by women mostly in the informal sector.

According to Baidoo and Parry-Hanson Kunadu (2018), *brukina* is a whole meal based on its rich nutrient and high energy. The production of *brukina* is done by mixing milled millet dough with fermented raw cow milk and sugar to taste.

Figure 7; *Brukina* (a diary product)

Millets to be used for the *brukina* is sorted out to eliminate stones and other unwanted materials which are washed thereafter. It is then dried and pounded to become powdery. A salt solution is sprinkled on the millet powder and mixed to form millet dough. It is then steamed and allowed to cool. A masher is used to break the dough into granules. To get a uniform granular shape, margarine or cooking oil is rubbed on the granules.

The fresh cow milk to be used for the *brukina* is pasteurized and allowed to cool. It is then allowed to sit for about 3days for natural fermentation to take place or it is inoculated with an already existing fermented milk. Due to the unavailability of constant supply of fresh

milk, some processors have resorted to using powdered milk. They mix the powdered milk with water to become liquid. It is then inoculated using a starter culture for fermentation.

A quantity of the processed millet dough is mixed with a portion of the fermented milk to get the *brukina* drink as shown in fig. 8. In recent times, Fanice (a product of Fanmilk Gh. Ltd) has further improve the taste of *brukina*.

In rural areas *brukina* is sold in calabash and tied in transparent rubber packages and sold in the open market. In the urban and semi-urban areas, it is packaged in polyethylene terephthalate (PET) bottles and refrigerated or ice blocks are put on them to keep them chilled.

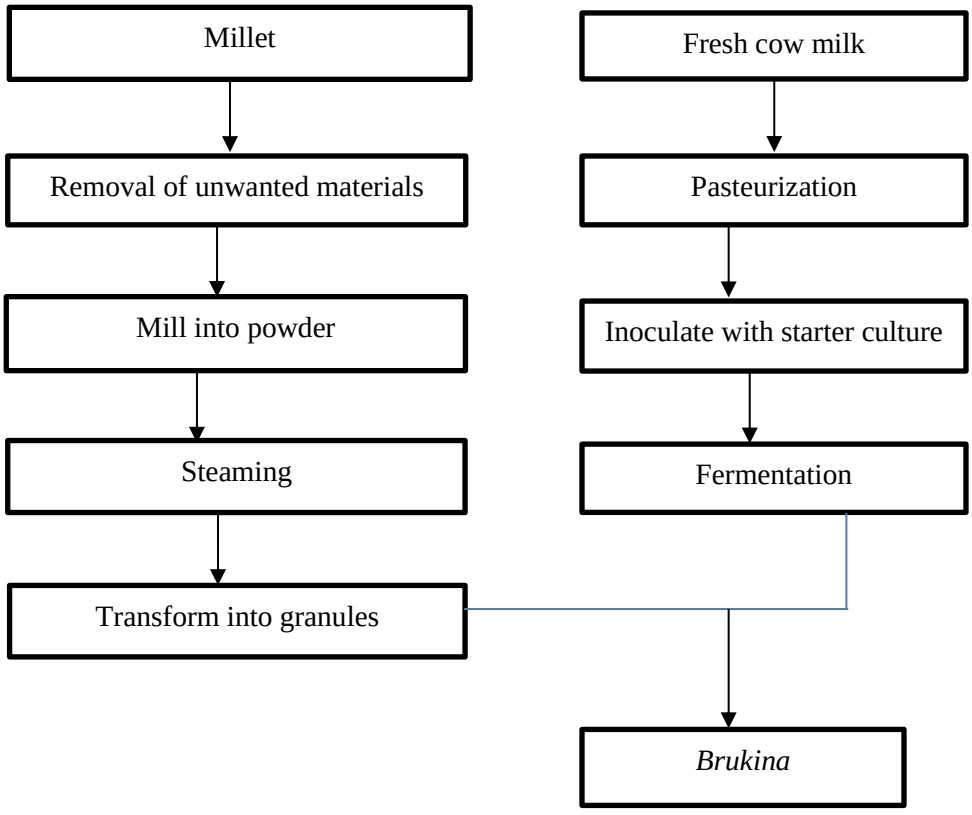


Figure 8: Flow diagram for *Brukina* production

2.3.2 The Local Diary Value Chain

The local diary value chain in Ghana consists of a wide variety of people made up of farmers, milk aggregators, wholesalers, retailers, local cheese makers and commercial cheese makers as summarized in table 7. Omore *et al.* (2004) explained that in the simplest form, the raw milk is sold directly to consumers without the use of any intermediaries but in extreme cases there are as much as three intermediaries between the farmer and the final consumer and these intermediaries are made up of rural assemblers, large urban assemblers and small fixed retailer or hawker. Some of the milk is also conveyed primarily to nearby traditional milk processors e.g. *brukina* and *wagashi* processors, who produce these products from their homes. As a common business practice, those sold directly to consumers have higher returns than when it passes through intermediaries. The more the intermediaries the lesser the return to the farmer and the costlier it becomes to the consumer.

Stockmen who own or raise the cow convey fresh milk day by day to their clients. Stockmen who do not have set up clients or who could not market their milk regularly amassed at a focal point of town to offer their milk for sale. Such places in Ghana include Tulaku, Nima, Ashaiman where fresh raw milk is sold together with boiled milk and other naturally fermented dairy products (Omore *et al.*, 2004). Owing to the rising prices of transporting the huge volumes of fresh raw milk from the rural areas to the urban areas, the profit margins for this business is very low in some instances and to compensate for this the raw fresh milk is adulterated with water to increase the volume.

The processing of local cheese (*wagashi*) used to be the main focus of a lot of local processors until the preference for *brukina* increased in recent years. *Wagashi*, *brukina* and

nunu are cottage industry products made by predominantly women who process just a small quantity of fresh raw milk. Most of these women have no formal education and learnt the trade as knowledge passed on in the family.

The local diary milk products are marketed and distributed in the informal market which is unregulated. The informal market is small-scale and has no incorporated cold storage systems because of the huge cost involved in procuring cold storage systems.

There are a number of institutions in the formal sector that produce dairy products in Ghana. The University of Ghana has a livestock and Poultry Research Center, which has a dairy unit that processes drinking and set yoghurt for sale on its campus. The Kwame Nkrumah University of Science and Technology (KNUST) has the Beef and Dairy Research unit under the school of Agriculture that manufactures yoghurt, cheese, butter and pasteurized milk, which are marketed on the university campus. There is also the milk collection center at Sekyere Odumasi that buys the fresh raw milk from the herdsmen and processes them into condensed milk and yoghurt. These formalized processing centers are in the middle belt of the country.

Table 7: Actors in the local dairy value chain (Omore *et al.*, 2009)

Actor Name	Summary description
Suppliers (Input)	Various: kraal materials and equipment, storage equipment (steel buckets, plastic containers and calabash). Because the cattle are allowed to graze openly, the most important input supply is materials for the kraal.
Farmers	Farmers can be classified as small scale (herd of 1-5 cows), medium scale (herd of 6-20 cows) and large-scale farmers (herds more than 20 cows). About 95 % of the farmers are men. The small and medium scale farmers play dual roles as both farmers and processors. They usually process their own milk for domestic consumption and sell some of the milk to the public. The largescale farmers sell their milk to medium sized dairy processors.
Assemblers or aggregators	The assemblers gather the fresh milk from the small and medium scale farmers and sell to retailers, wholesalers or processors. They are mostly male dominated.
Processors	Milk is processed both the home level as well as in industry. At the household level it is processed into *wagashie, nunu, fura* and *brukina.* At the industrial level it is processed by mostly cottage industries who process them into mostly yoghurts and fresh milk. The cottage industries are able to package and brand them because of their good knowledge in marketing.

Retailers and Distributors	About 80% of the dairy products are sold directly to retailers from the initial processers (farmers), others are sold on the local market directly to consumers. The cottage industries sell their products to retailers including supermarkets, mini marts and local stores

In the southern parts of the country, the Ministry of Food and Agriculture has established the Amrahia Dairy Farm that collects milk from milkers and process them into yoghurt and dairy products. The Nungua Farms which is under the control of the Animal research Institute also process small quantities of milk into yoghurts.

Concerning the large-scale processing of dairy products, Nestle Ghana Limited, Promasidor Ghana limited and FanMilk Ghana Limited play key roles. Though they do not use fresh milk from the local market, they import huge quantities of powdered dairy to produce various products like Ideal milk which is an evaporated milk, fan ice (yoghurt-based ice cream), cowbell (powdered milk) and a host of other products.

2.3.3 Possible causes of milk adulteration

One of the main problems that results in poor status of milk in the local sector is contamination from the farm. Soomro *et al*., (2002) indicated that the rich moisture content as well as nutrient composition helps in facilitating the growth of microorganisms. Unhygienic conditions and human factors mentioned below play the leading roles in contamination of milk.

Farmers do not have designated places for expressing milk from the cow. Milking is expressed in the open exposing the milk to opportunistic pathogens, dust and insects. Some of these insects and flies are mostly in the environment because of the droppings of the cattle which makes the surroundings unhygienic. The hind legs and the tail of the cattle are also not tied, resulting in the cow beating its tail and moving its hind legs thereby introducing dust and particles into the milk during milking. Dilielo, (1982) and Atiqah, (2017) also made a point that under the above conditions, various microorganisms can find their way into the milk collected from the cow. Freedman, (1977); Owusu-Kwarteng *et al.*, (2018) also adds that sources of contamination includes the farm environment and the utensils that are used to store the milk on the farm the transportation of the milk and dairy products to the market.

Nguyen and Sperandio (2012) have indicated that young cattle have become the hub of *Escherichia coli*. This was supported by Desmarchelier, (2011) who added that enterohemorrhagic *E. coli* (EHEC) is a dangerous type that is often linked to food borne diseases caused by dairy products and lead to hemorrhagic colitis which is also known as bloody diarrhea. The risk of EHEC occurs by ingesting contaminated milk products as indicated in table 8.

Transmission of *E. coli*, to human has been identified to be from animal. Both transmission forms have led to the outbreaks of diseases. Animal-to Person transmission comes as a result of playing with animals and handling animal products contaminated with the microbe (Bélanger *et al.*, 2011). Transmission from one person to the other is through fecal contamination to the oral route which is mostly identified in young children and also infected food handlers. Martin *et al*. (1986) and Pal (2017) buttressed that that the most

likely course of transmitting *E. coli* to persons is through fecal contamination of milk which has often resulted in the outbreak of gastroenteritis.

Table 8: Dairy products contaminated with diarrhoeagenic *E. coli*

Product	Prevalence
Raw cow's milk	10.7%
Raw camel's milk	33.3%
Raw goat's milk	28.6%
Fermented cow's milk	25%
Cheese	42.9%
Ice cream	0%
Curd	32.58%
Yoghurt	4.35%
Déguè	16.09%

Source: (Aboubaker *et al*., 2016; Bagré *et al*., 2017)

In the Ghanaian context, a prime factor that results in poor quality of milk and its contamination is the crude methods of milking. Cows can be milked using their hands or machines but the former pertains widely in Ghana. Sampane-Donkor (2002) found that, the milkers introduce microorganisms into the milk when using their hands to milk the cows and they do this by not observing aseptic techniques (Liu *et al*., 2018). Most of them do not make sure their hands as well as the udder of the cows are washed before expressing the milk and also use already contaminated utensils to collect the milk. Contamination is also introduced through wholesalers and herdsmen who processed their

own milk in the value chain (Sampane-Donkor, 2002). It is therefore necessary to maintain good hygiene in handling milk products. This is necessary because after milking certain insects may have landed on the utensils to feed on some milk droplets in the utensils introducing pathogens into it.

Mwini and Darkwa (2016) observed that the producers of *wagashi* did not refrigerate the leftovers. Most of them were also ignorant of the availability of health certificates which are given to the local processors who follow strict hygiene processes. Milk is highly perishable so when it is not transported to the vending sites on time and at the ambient temperatures, microorganisms present tend to multiply rapidly. Further, some of these milk products are exposed during vending to attract customers. This practice exposes the milk products to environmental microbes.

2.4 Milk Pasteurization

Pasteurization is when heat is used during processing of food at high temperatures kills bacteria that are pathogenic or non-spore forming organisms to make the food safe to eat (Ellingson *et al.*, 2005). To eliminate microorganisms in milk, a minimum temperature and time is required. Table 9 shows various times and temperatures for milk pasteurization. Kammerlehner (2009) reported that effective milk pasteurization temperature and time is about 72℃ for 15 seconds. Unfortunately, these standards are not enforced in the production of indigenous milk products vended in the local markets where the possible danger of zoonoses may exist as stated by Donkor *et al.,* (2007).

Table 9: Combinations of time and temperature in milk pasteurization

Pasteurization temperature°C	Time
63	30 min
72	15 s
89	1s
90	0.5 s
94	0.1 s
96	0.05 s
100	0.01 s

Source: (Hayes *et al.*, 2001)

2.5 Morphology of *E. coli*

Figure 9: A micrograph of *E. coli* (Source: https://www.britannica.com/science/E-coli, 2016)

E. coli a rodlike-shaped bacteria of size ranging from 0.7 to 1.5×2 to 5µm which occurs singly or in pairs ,it is as well as gram-negative bacteria (Cooke, 1974; Reshes *et al.*, 2008; Aryal *et al.*, 2016). *E. coli* is a facultative anaerobe which makes it a suitable occupant of the lower intestine of warm-blooded animals (Aryal *et al*., 2016). All *E. coli* have cells endowed with a three-layered structure namely the cytoplasm, peptidoglycan and an outer membrane. The rigid nature of the peptidoglycan confers the rod shape of the *E. coli* (Reshes *et al.*, 2008). *E. coli* is non-sporing and may possess flagella depending on the strain, which facilitate its motility (Aryal *et al*., 2016; Hendriksen, 2003). The flagella possess the heat and alcohol labile H- antigen, which is one of the four antigens of *E. coli*. The other antigens include O (somatic), K (capsular) and F (fimbria) which are situated in the surface of the outer membrane, the envelope and the fimbriae, respectively (Aryal *et al*., 2016).

E. coli morphology is recognized as either, rough, flat, and irregular or smooth, high and circular (Hasman *et al.*, 2000). The chemical properties of the media used to culture the organism determines the appearance of the organism in cultured. For instance, on MacConkey agar, *E. coli* isolates produce bright pink colonies, with an intense yellow-green peripheral zone on Brilliant Green and on Eosine-methylene blue (EMB) agar, they produce characteristic metallic sheen colonies (Zinnah *et al*., 2007).

2.6 Prevalence of diarrhoeagenic *E. coli*

E. coli is normally regarded as an organism that is harmless, nevertheless, some gastrointestinal ailments, neonatal meningitis, urinary tract infection, and gastroenteritis is informed to caused diarrhoeagenic *E. coli* via the transmission of *E. coli* in contaminated

foods or water (Croxen and Finlay, 2010). This implies that the occurrence of *E. coli* in food is of significant public health interest due to the presence of Enteropathogenic and/or toxigenic strains (Claeys *et al.*, 2013). Although many microorganisms can ingress milk and milk products, the presence of *E. coli* is regarded as a definitive indicator of indirect or direct fecal contamination (Oliver *et al.*, 2009). Brooks *et al.*, (2018) stated that according to the Center for Disease Control and Prevention, 9 % of foodborne disease outbreaks in the United States were attributed to the consumption of dairy products. Shiga toxin-producing *E. coli* caused 6 % of the outbreaks and 3 % of the illnesses associated with food contamination (Brooks *et al.*, 2018). *E. coli* O157:H7 has been incriminated in Hemolytic Uremic Syndromes (HUS). (Bae and Lee, 2017; Chen, 2015; Luciani *et al*., 2016).

Diarrhoeagenic *E. coli* is a major public health concern in both the developed and less developed countries. Several studies have reported on the presence of diarrhoeagenic *E. coli* in the food industries with specifics in the meat and dairy sub sector (Huang *et al*., 2014; Ateba and Mbewe, 2011; Mason *et al.*, 2009; Obi *et al*., 2004); Aworh *et al*., 1987;) and have confirmed the rate at which the infections of diarrhoeagenic *E. coli* impacts the human body negatively.

The prevalence of diarrhoeagenic *E. coli* has been reported to be about 1-33.5 % in raw cow milk samples around the world (Altalhi and Hassan, 2009; Gomes *et al*., 2016). However, the occurrences of diarrhoeagenic *E. coli* in raw cow milk in advanced countries like Australia, Canada, France, and USA have been reported to be significantly lower at 1.3 %, 2.3 %, 3.2 %, 3.6 %, and 2.4 % respectively (Kai and Aotearoa, 2009; Johnson *et al.*, 2005; Schlesser *et al*., 2006).

Ombarak *et al.,* (2016) reported the occurrence of *E. coli* in raw milk, Ras cheese and Karish cheese in Egypt at a prevalence of 76.4 %, 74.5 % and 21.7 % respectively. Ombarak *et al.,* (2016) found 222 strains of *E. coli* from 187 different milk samples they observed. The remaining 69 (36.9 %) of the samples had one or more virulence gene which constituted 46.9 % of the 69 samples. Ombarak *et al.,* (2016), however, indicated that a high number of the potentially virulent *E. coli* isolates identified in the study were not in the clinically important O serogroups, with the exception of O75, O91, and O166 which have been linked to human diseases.

A research conducted in West Australia in 2007 which sampled 183 milk products reported a 90 % prevalence of *E. coli* in the samples (Kai and Aotearoa, 2009). This suggests that milk samples especially raw milk that are not handled and processed under hygienic conditions exhibit the capability of posing some health risks to consumers.

Similarly, the findings of Soomro *et al*., (2002) indicated that the presence of *E. coli* was 57 % in 100 raw milk samples and 51 % in 60 milk product samples in Tandojan, Pakistan. Soomro *et al.,* (2002), attributed the high prevalence of *E. coli* in the dairy products to unhygienic methods of production, transporting, handling and sale of the products. On the contrary, Chaleshtori *et al.,* (2017) found a much lower prevalence of *E. coli* in indigenous milk product in Kashan, Iran. Chaleshtori *et al.,* (2017) reported that out of the 60 ice cream, 30 yogurt and 26 cheese samples, *E. coli* was present is 8.33 %, 10 % and 11.54 % of the samples respectively. Chaleshtori *et al.,* (2017) indicated that the occurrence of *stx1* and *stx2* was both 9.4 %. A similar finding conducted in the Van province in Turkey was reported by Sancak *et al.,* (2015) that *E. coli* O157:H7 was present in 11 % and 6 % of 100 raw milk and 100 herby cheese samples, respectively.

Oliver *et al.,* (2009) also reported 0.075 % and 2.4-3.96 % prevalence of *E. coli* O157:H7 and Shiga-toxin, respectively from bulk tank milk and milk filters. Sudda *et al*., (2016) reported that the occurrence of pathogenic *E. coli* in 16.7 % of 65 raw milk samples from zero-grazed cows in Arusha city, Tanzania. Sudda *et al*., (2016) further indicated that the mean Colony Forming Unit (cfu) of the *E. coli* was 2 cfu/ml. Sudda *et al.,* (2016) explained that the low prevalence of *E. coli* could probably be attributed to the collection of the milk from apparently healthy cows.

On the contrary, Brooks *et al.,* (2012) who studied the microbial quality and prevalence of food borne pathogens of 41 raw milk aged cheese samples found no *E. coli* present in the sampled products. This report supported the findings of Paneto *et al.,* (2007) who found only one sample positive for *Listeria monocytogenes* out of 181 raw milk aged cheese. According to Soomro *et al*., (2002) regularly sterilizing equipment, cleaning of utensils, proper washing of milker's hands and udders could help drastically to reduce the prevalence of microorganisms and other pathogenic microorganisms in raw milk and milk products. In addition, eradication of diseased animals, pasteurizing/ boiling of milk prior to collection and distribution for consumption and product making could consequently reduce the food-borne diseases associated with milk.

2.7 Pathogenicity of *E. coli*

Pathogenic *E. coli* are grouped into 8 based on their properties of virulence, mechanism of pathogenicity and clinical syndrome (Kai and Aotearoa, 2009; Olsvik *et al.,* 1991). These groups include Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enteroaggregative *E. coli* (EAEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli*

(EIEC), Diffusely Adherent *E. coli* (DAEC), Uropathogenic *E. coli* (UPEC) and Neonatal Meningitis *E. coli* (NMEC) (Croxen and Finlay, 2010; Kai and Aotearoa, 2009; Saxena *et al.,* 2015). These 8 pathovars are further classified into 2 broad groups, namely diarrhoeagenic *E. coli* which consist of the first six pathovars listed above and Extraintestinal *E. coli* (ExPEC) consisting of the last two pathovars (Croxen and Finlay, 2010).

Following ingestion, these pathovars with the exception of EIEC adhere themselves to specific host cells with the aid of long appendages called fimbriae or pili (Croxen and Finlay, 2010). These *E. coli* strains overcome the normal functioning of the host cells with the aid of secreted proteins. Each pathovar has a unique way of adhesion, invasion and colonization of host cells which ultimately results in the disease noted for that pathovars or strain (Croxen and Finlay, 2010; Desmarchelier, 2011).

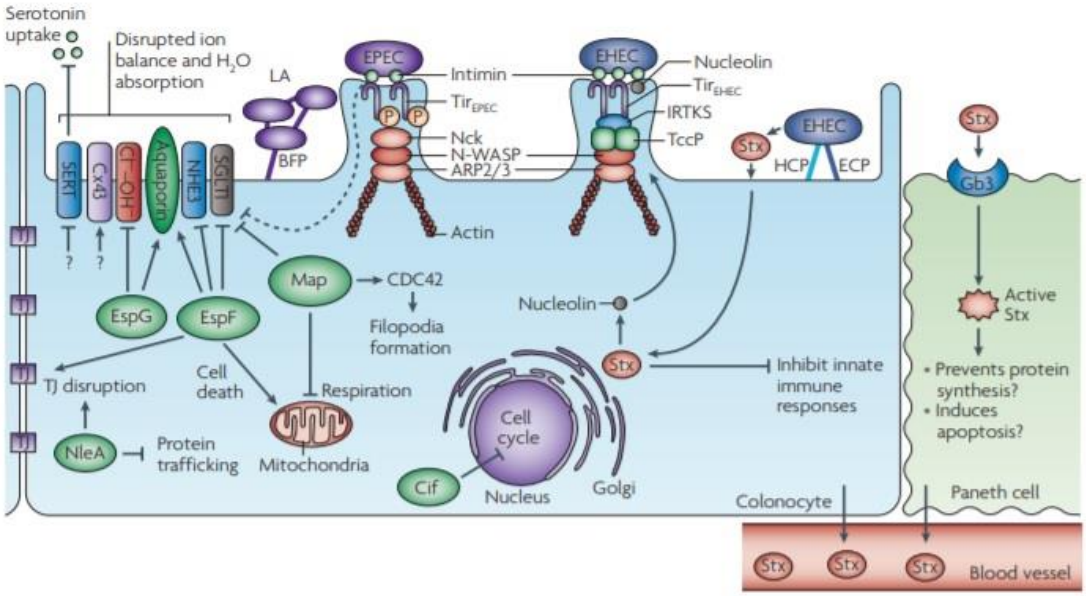
2.7.1 Enterohemorrhagic *E. coli* (EHEC)

This pathovar is normally present in the feces of healthy cattle, suggesting that their prevalence in raw milk is an indication of direct or indirect fecal contamination (Kai and Aotearoa, 2009). Nevertheless, Kai and Aotearoa, (2009) indicated that in cases of mastitis infection in the cattle, EHEC strains can be excreted via the udder.

EHEC are commonly referred to as Shiga-toxin producing *E. coli* (STEC), Shiga-like toxin producing *E. coli* (SLTEC) or Verocytotoxin-producing *E. coli* (VTEC) (Chaleshtori *et al*., 2017; Desmarchelier, 2011; Kai and Aotearoa, 2009). These strains are incriminated in most severe outbreaks of gastroenteritis in developed countries (Croxen and Finlay, 2010). The common serotype amongst these pathovars of *E. coli* is O157:H7. Findings of Brown

et al. (1997) indicated that the intrinsic tolerance of pH is as high as 3.0 for *E. coli* O157:H7. The symptoms of their infections extend from milk diarrhea to hemorrhagic colitis and the fatal hemolytic-uremic syndrome (HUS) (Desmarchelier, 2011).

The pathogenicity of EHEC strains is centered on their unique capability to produce one or two cytotoxins known as Shiga toxins (*stx1* and *stx2*) and the ability to produce the protein called intimin, a virulence factor that is responsible for strong attachment of the EHEC strain to host intestinal epithelial cells (Chaleshtori *et al*., 2017; Kai and Aotearoa, 2009). As shown in fig. 10, the intimate attachment of the strain to the host cell as a result of the intimin, which is encoded by the chromosomal gene *eaeA,* leads to attaching and effacing lesions in the intestinal mucosa (Chaleshtori *et al*., 2017; Kai and Aotearoa, 2009). According to Desmarchelier (2011), most of the strains that cause hemorrhagic syndromes are enhanced by the presence of the locus for enterocyte effacement (LEE) located on the chromosomal pathogenicity island. Apart from the popular O157:H7, others O26, O111, O45 and O103 are some of the EHEC serogroups of clinical significance. These serotypes often carry the same virulence characteristic such as *stx1* and *stx2* and the LEE.



Source: (Croxen and Finlay, 2010)

Figure 10: Pathogenic mechanisms of enteropathogenic and enterohaemorrhage *E. coli*

2.7.2 Enteropathogenic *E. coli* (EPEC)

This pathovar is highly implicated in most acute or chronic enteritis in children in developing countries (Desmarchelier, 2011). Like EHEC, EPEC form attaching and effacing lesions in the intestinal epithelium encoded by *eaeA* genes bearing in the 60MDa EPEC adherence factor (Desmarchelier, 2011; Kai and Aotearoa, 2009). However, according to Croxen and Finlay, (2010) attachment of EPEC strains to host cells is aided by the bundle-forming pili which aids in the formation of microcolonies via the interaction with other EPEC and also interact with N-acetyl-lactosamine-containing receptors on host cell surfaces. Upon invasion of the host cells, a large reposition of effectors are translocated by the Type three secretion system (T3SS) into the host cells that results in cytoskeletal rearrangements and immune modulation and contribute to diarrhea (Croxen and Finlay,

2010). According to Croxen and Finlay (2010), the presence of the proteins EspB and EspF have been shown in the inhibition of phagocytosis, whiles the proteins EspJ also inhibits opsonophagocytosis by red blood cells. The time for the onset of illness is within 17–72 hours and the duration of illness is from 6 hours to 3 days. The bacteria cause illnesses with symptoms such as fever, vomiting, abdominal pains, and severe diarrhea in infants, which may persist for more than 14 days. In adults, nausea, vomiting, severe watery diarrhea with prominent amounts of mucus but without blood, headache, abdominal cramps, chills and fever is mostly encountered.

2.7.3 Enterotoxigenic *E. coli* (ETEC)

This pathovar has been incriminated as the most common cause of traveler's diarrhea (Kai and Aotearoa, 2009; Olsvik *et al.*, 1991). According to Liaqat and Sabri (2010), consumption of ETEC-contaminated food is the most common cause of diarrhea and can have fatal consequences in children under the age of 5.

The main virulence factor of ETEC include the fimbriae which assist in the colonization of the intestine and the secretion of the enterotoxins, heat-stable enterotoxins (STS) and heat-liable enterotoxins (LT) (Croxen and Finlay, 2010; Desmarchelier, 2011; Kai and Aotearoa, 2009). According to Kai and Aotearoa (2009), ETEC resembles the cholera toxin both in structure and mode of action, interfering with water and electrolytes movement across the intestinal epithelium. Strain attachment to host cells may be achieved by the outer-membrane proteins Tia and TibA (Croxen and Finlay, 2010). The enterotoxins produced by the ETEC causes diarrhea which results in poor absorption of Na^+ and H_2O efflux into the lumen.

EatA which is also one of the virulence factors of the ETEC known to be a serine protease autotransporter of the Enterobacteriaceae (SPATE) that clears Cathepsin G had also been reported to increase fluid build-up (Croxen and Finlay, 2010). The excess accumulated fluid is evacuated as watery diarrhea when the volume of the accumulated fluid exceeds the normal absorption capacity of the large intestine (Kai and Aotearoa, 2009).

2.7.4 Enteroinvasive *E. coli* (EIEC)

This pathovar, unlike other pathovars, includes obligate intracellular parasites that have neither flagella nor adherence factors. Virulence is elicited by a 220kb plasmid that encodes a T3SS on the Mxi-spa locons hat is needed for invasion cell survival and apoptosis of macrophages (Croxen and Finlay 2010). However, according to Desmarchelier (2011), pathogenicity of EIEC is linked with a plasmid-encoded type III secretory apparatus and other plasmids encoded virulence factors. Pathogenesis of EIEC is similar to that of *Shigella*.

Following the invasion of the intestinal epithelial cells of the host where they multiply, EIEC causes cell destruction and acute inflammatory response (Kai and Aotearoa, 2009). Humans are the main reservoirs of EIEC, suggesting that outbreak is associated with the consumption of food and water contaminated with human feces or person-to-person transmission (Desmarchelier, 2011).

2.7.5 Diffusely Adherent *E. coli* (DAEC)

This pathovar has been reported to cause diarrhea in children between the ages of 18 months and 5 years as well as in recurring urinary tract infections (UTIs) in adults (Croxen

and Finlay 2010). DAEC strains adhere to Hep-2 tissues via Afa-Dr adhesion interactions (Desmarchelier 2011). According to Desmarchelier, (2011) secreted autotransporter toxin (Sat) has been incriminated in lesions of tight junctions that are formed with Afa-Dr DAEC infection and increased paracellular permeability.

2.7.6 Enteroaggregative *E. coli* (EAEC)

This is the second most frequent cause of travelers' diarrhea. The diarrhea is mostly watery, but it can contain mucus or blood (Croxen and Finlay, 2010). Like DAEC strains, EAEC strains distinguishably adhere to Hep-2 tissues cells in a distinct aggregative or "stacked-brick" pattern (Desmarchelier, 2011; Kai and Aotearoa, 2009). According to Croxen and Finlay (2010), EAEC strains attached to enterocytes in both the small and large bowels via aggregative adherence fimbriae (AAF) that trigger a strong inter-lenkin-8 (IL-8) response, permitting the formation of biofilms in the cell surfaces. EAEC biofilms, unlike the biofilms of other nonpathogenic *E. coli,* are embedded in a thick mucus layer (Croxen and Finlay, 2010). The EAEC strains secreting cytotoxins to cause impairment in the mucosal.

2.8 Isolation and identification of diarrheagenic *E. coli*

Separation and detection of *E. coli* mostly employ traditional culture-based methods involving initial enrichment, selective enrichment, biochemical screening and serological confirmation. Tryptone Soy broth (TSB) and *E. coli* broth (EC) are the two most frequently used culture media for detection of *E. coli* O157:H7 and VTEC serogroups (Saxena *et al*., 2015). Other selective and differential media as shown in table 10 have been used to culture *E. coli* O157:H7 based upon the fact that it lacks beta-glucuronidase activity.

Table 10: Technologies	**for detecting *E. coli* O157:H7**
1	Latex agglutination
2	Enzyme-linked Immunosorbent (ELISA).
3	Colony Immunoblot Assay
4	Direct immunofluorescence filter
5	Immunocapture technique
6	Monoclonal antibodies
7	Polyclonal antibodies

Sourced:(Boer, 2000)

Sorbitol MacConkey agar (SMAC) alongside cefixime with either potassium tellurite or rhamnose are the most frequently used selective media. The inability of *E. coli* O157:H7 to ferment sorbitol enhance its detection on stool cultures as it produces colorless, opaque colonies on SMAC, unlike non *E. coli* O157:H7 strains which produces pink colonies on SMAC. This is a characteristic quality of most other pathogenic diarrhea inducing classes of *E. coli* such as ETEC and EPEC (Johnson *et al*., 2005). Saxena *et al.* (2015) informed that the use of hemorrhagic colitis (HC) agar and commercial chromogenic agars like

rainbow agar for the detection of EHEC O157:H7. There are several biochemical tests as indicated in Table 11, which are used to confirm the presence of *E. coli.*

Table 11: Biochemical reactions of *E. coli*

Biochemical Test	**Reaction**
Catalase	+ ve
Simmon's citrate	-ve
TSI	A/A +gas
Gelatin liquefaction	-ve
Indol Production	+ ve
Nitrate Reduction	+ ve
Urease	- ve
Voges Proskaur	- ve
Methyl Red	+ ve
Presumptive test	+ ve

Sourced: (Soomro *et al.*, 2002)

In some cases where serially diluted samples are cultured on MacConkey, to observe the growth of colonies, an isolated colony is picked again from the agar and cultured once more on the MacConkey agar to obtain purified isolates (Soomro *et al.*, 2002). Subsequently, a confirmation test is done by culturing pure colonies on other media as in table 10.

According to Liu *et al*., (2018), *E. coli* O157:H7 specificity in relation to culture-based methods is low because some pathogenic organisms like *Citrobacter frenndii* share same antigen with it, leading to cross-reactions and untrue optimistic outcomes.

It necessitated advancement of rapid methods like PCR based assay, isothermal amplification assays and antibody-based immunoassays for *E. coli* detection, which have been reported to exhibit good sensitivity and specificity (Saxena *et al.*, 2015; Wu *et al*., 2015; Luciani *et al.*, 2016). However, PCR based methods involve exclusive apparatus, trained workers and cannot differentiate viable cells from dead cells (Owusu-Kwarteng *et al.*, 2017; Wu *et al*., 2015; Zhang *et al*., 2017; Chen *et al*., 2017). This suggests that cells that are dead can give incorrect positives PCR outcomes, which could result in unnecessary product recalls and economic losses. ELISA and other immunoassays which rely on the antibody for *E. coli* are expensive, sensitive to matrix effect and difficult to store and transport (Wu *et al.,* 2015). The high-resolution melt curve (HRM) based PCR assay is highly sensitive to any single base change in enlarged targets (Liu *et al.*, 2015). HRM dyes are able to completely saturate amplicons, this yielding a melt curve with higher resolution (Liu *et al.,* 2018). The use of HRM in the areas of clinical diagnostic and food safety according to Liu *et al.*, (2018) is on the rise owing to its simple operation, comparatively cost less and effortlessness of use.

Although the old-style methods for *E. coli* recognition are relatively sensitive, of low cost and enhanced the ability to monitor cell viability, these methods are tedious and laborious and requires longer time (3days) to obtain confirmatory result (Wu *et al.*, 2015; Zhang *et al*., 2017).

2.9 *E. coli* adaptation

Food processing procedures of recent years have tended to a less aggressive processes, including the use of high hydrostatic pressure, irradiation in smaller doses and heat application that is mild as compared to the old-style treatment like pasteurization and sterilization. The modern methods of food processing aim to maintain the textural, sensory and the initial characteristic of fresh food products. Nevertheless, due to the less lethal effect of the applied conditions, cell increase as well as adaptation of microorganisms might not be hindered (Velliou *et al*., 2011).

Microorganisms like *E. coli,* show stress adaptation which is the increased resistance of the bacteria to lethal ecological conditions (Velliou *et al*., 2011). However, an exposure of the microorganism to one kind of stress may result in the development of resistance to another kind of environmental stress (Cabiscol *et al.*, 2000). For instance, an exposure of *E. coli* to sub-lethal acid stress result in cross-protection to other non-related environmental stresses (Parry-Hanson *et al.*, 2009b). Most industrial preservation or manufacturing processes of dairy products depends on acidification techniques like fermentation to regulate the development and survival of the microorganisms (Parry-Hanson *et al*., 2009b).

E. coli can acclimatize to surroundings that are acidic in different ways. According to Foster (2001), they are able to avert entry of protons in the cell as well as protect and repair their damaged macromolecules by making the cells free from protons once their inner pH gets to a perilous acidic pH. He also adds that they have the capabilities of make the pH optima in the acid range to enable them function normally when their internal pH falls. Slonczewski *et al.* (2009) also adds that one of the mechanisms used by *E. coli* for pH

homeostasis is K^+ transport which they use to compensate for the proton extrusion by alkalinizing the cytoplasm at low external pH.

E. coli O157:H7 has been detected in foods that are acidic like dry fermented sausages according to reports by Bae and Lee, (2017) and O'Bryan *et al.*, 2017). According to Fink (2018), when microorganisms encounter environmental stresses, they respond via the production of proteins called shock proteins; for example, GroEL, DnaK, DnaJ, which repair the injuries or damages caused by the stress factors.

The change in the lipids located in the membrane are significant in ensuring the integrity and functionality of the membrane amidst external perturbation according to Elhadidy and Álvarez-Ordóñez (2016). Also Álvarez-Ordóñez *et al*. (2013) observed a similar occurrence for some *Salmonella spp.*

Report of Brown *et al.* (1997) indicated that acid adapted *E. coli* had a significant decrease in the total log CFU, although the reduction was more gradual as related to the *E. coli* that had no adaptation with acid which decreased rapidly during a 4-hour challenge test. According to Velliou *et al*. (2011), the induced hinderance rest on the amount and type of acid used, suggesting that varying degree of acid used and the types of acid used results in different levels of induced heat resistance.

This observation was also consistent with the findings of Ryu and Beuchat, (1998) and Sainz *et al.*, (2005) who indicated that *E. coli* that has been adapted with acid had better growth and survival in acidic foods. In contrast, Parry-Hanson *et al*. (2009b) reported low growth for *E. coli* cells that has been acid-adapted as compared with cells of *E. coli* without any adaptation of acid in fresh goat milk (pH 6.9). This finding was in agreements with the

findings of Hsin-Yi and Chou, (2001) who observed decline in growth of acid-adapted cells of *E. coli* associated with cells *E. coli* O157:H7 that have no adaptation of acid in diluted fermented milk known as Yakult (pH 3.9) as well as yoghurt (pH 3.9) at a temperature of 7°C for a period of 6 days. Parry-Hanson *et al.* (2009b) explained that even though acid-adaptation triggers protection the process may stress the cells of *E. coli* O157:H7 therefore the organism's sensitivity may increase whiles enhancing its survival. Parry-Hanson *et al.* (2009b) further explained that energy is applied in maintaining the adaptation of *E. coli* O157:H7 cells so the organism may re-adapt to the acidity of its environment therefore influencing its growth.

Generally, the use of hurdle effect where more than one (salt and acid) inhibitory agent is applied has been said to be more efficient (Bae and Lee, 2017). Combination of acid and salt treatment as a method of preservation is very common in the food industry. It is used in foods such as meat that has been fermented, cheese and salad dressing as well as sauce. According to Bae and Lee (2017) when acetic acid and salt are used in combination treatment, acetic acid is the main factor that inhibits the multiplication of the organism.

According to Lee and Kang (2016), what inhibits acetic acid effect declines when salt is added since low pH increases acid resistance or acid tolerance response so the organic acids inhibits the cytoplasm from being acidified. A similar observation of the antagonistic effect of acetic acid and salt was observed by Bae and Lee (2017) in Luria-Bertani (LB) broth. However, Bae and Lee (2017) observed a synergistic hindering outcome on *E. coli* in phosphate buffer, buffered peptone water and peptone water. The LB broth employed by the researchers contained an extract of 0.5 % yeast in addition to 1 % tryptone. Bae and Lee (2017) explained that the incompatible outcome of the salt and acetic acid constituent

in the LB broth could be ascribed to the involvement of organic compounds. Some of the organic compounds are yeast extract and tryptone which have the compatibility to convene a protecting result alongside salt contradicting the treatment of acetic acid.

Bae and Lee (2017) also indicated that when either Na^+ ions or Cl^- ions are added following the treatment of acetic acid, increases survival of *E. coli* O157:H7. A similar observation reported by Casey and Condon (2002) established that the ions of Na as well as Cl offer partial defense distinctly in acidic pH. Therefore, hindrance outcome against *E. coli* O157:H7 would be observed if more than 2 % salt only is used (Lee and Kang 2016).

CHAPTER 3

3.0 METHODOLOGY

3.1 Study Area

The samples were collected from five locations (Tulaku, Ashiaman, Nima, Dzorwulu and Madina) in Greater Accra, Ghana (fig. 11). These places were selected based on the occupation of most of the people residing in the area and the market places and streets where these dairy products are sold. Cattle rearing and milk production are dominant occupations of inhabitants in some of the selected localities.

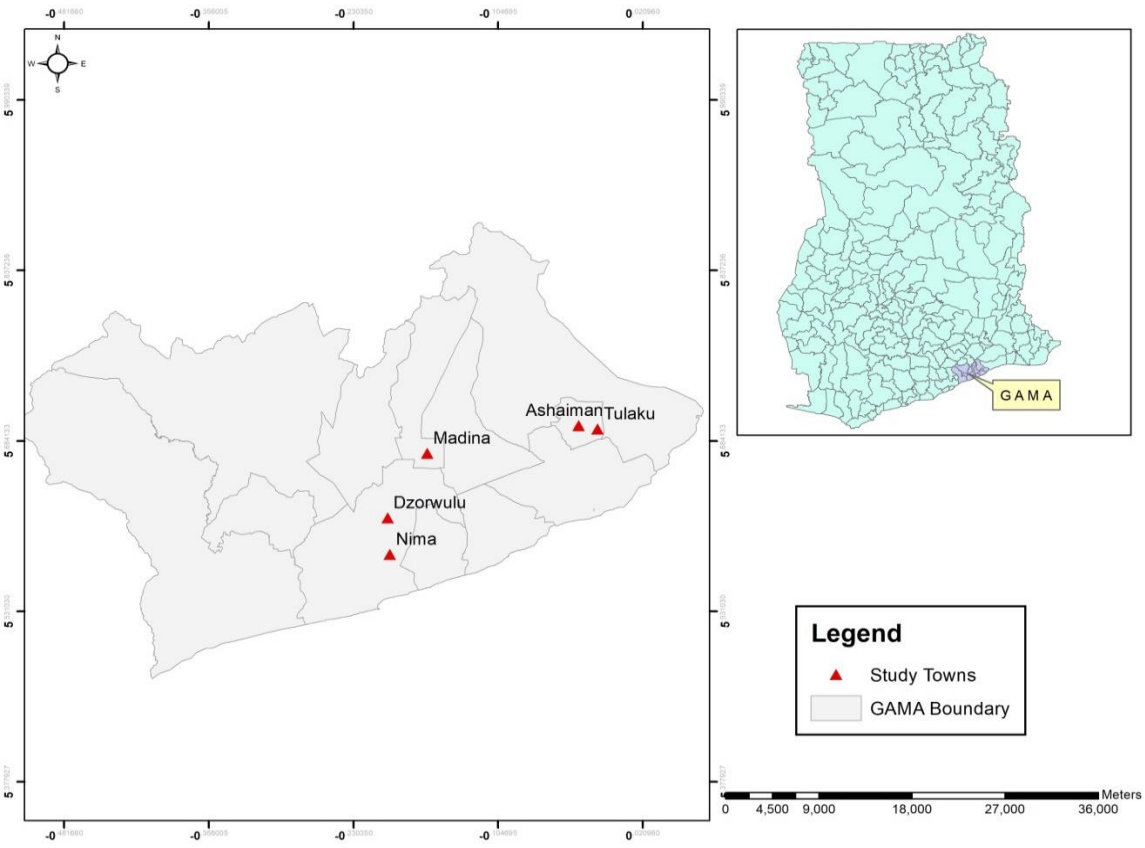


Figure 11: Map of the research area

3.2 Sample Collection

An average of 300 ml of informal fresh milk products (raw milk, boiled milk, *brukina, nunu* and *wagashi*) were conveniently purchased from street vendors from each sampling site. Some samples (*brukina, nunu*) were in screw-capped bottles whiles others (raw milk, boiled milk, *brukina*, *nunu* and raw *wagashi*) were packaged in transparent polyethylene bags. All samples were labeled with permanent markers and kept below 4 $^{\circ}$C in an ice chest with frozen icepacks. Samples were then transported within 2 hours of purchase to the Microbiology Laboratory of the Department of Nutrition and Food Science, University of Ghana. At the laboratory, the samples were stored in the refrigerator at a temperature of 4 $^{\circ}$C for not more than 24 hours until microbiological analyses.

3.3 Isolation of *E. coli* from informal dairy products

3.3.1. Enrichment of *E. coli*

Before pre-enrichment, the milk samples in the bottles were shaken vigorously to enable adequate homogenization of samples. Primary enrichment was done by aseptically weighing 25 g of the milk sample into 225 ml of sterile Brain Heart Infusion (BHI) Broth (Oxoid CM1135, Basingstoke Hants, United Kingdom). The samples were blended in a stomacher (Stomacher 400 circulator, Seward Medical London, England) for 30 seconds at normal speed and the homogenates incubated for 10 minutes at room temperature with periodic shaking. The samples were allowed to settle by gravity for 10 minutes. The medium was then incubated at 37 °C for 3 hours to resuscitate injured cells (Fang, 2013).

All the inoculated primary enrichment broth was then transferred into 225 ml of a double strength Tryptone Phosphate Broth (TPB) (Oxoid CM0283, United Kingdom) prepared according to the manufacturer's instruction and incubated at 44 ℃ for 20 h (ISO 16649-1:2001).

3.3.2. Isolation

Samples from the enriched TPB broth were streaked on pre-poured MacConkey agar (Oxoid CM0007, United Kingdom) and Eosin Methylene Blue (EMB) agar (Oxoid CM0069, United Kingdom). Both media were prepared according to the manufacturer's instructions. The inoculated plates were incubated at 37^{o}C for 24 h to identify presence of *E. coli* in dairy samples.

3.3.3. Isolation of *E. coli* O157:H7 on Sorbitol MacConkey (SMAC) Agar

At least 10 presumptuous *E. coli* colonies from each MacConkey plate were streaked on SMAC agar (Oxoid CM1005, United Kingdom). The plates were incubated at 37 ℃ for 24 h. Samples that were presumptive positive for *E. coli* O157:H7 sorbitol negative and appeared colorless on SMAC plates.

3.3.4. Confirmation of *E. coli* O157:H7 with Antisera

All presumptive positive colonies of *E. coli* O157:H7 were further confirmed with the monovalent *E. coli* O157:H7 antisera following the manufacturer's instruction. Samples that were positive for *E. coli* O157:H7 formed agglutination after 5 mins.

3.4. DNA extraction and identification of diarrhoeagenic *E. coli* (DEC) virulence genes using Real-Time (RT) PCR

3.4.1. DNA Extraction

DNA was extracted directly from the dairy samples that were positive for *E. coli* by using the ZymoBIOMICS^{TM} DNA Miniprep Kit, following the manufacturer's instructions with slight modifications.

Briefly, the dairy samples were incubated for 48 hours at 4 ℃ and the samples except *wagashi* were centrifuged at 5000 xg for 5 min. The supernatant of each of the samples was discarded and the pellets were diluted with 1 ml sterile distilled water. The *wagashi* was measuring (5 g) into 15 ml of buffered peptone water and the sample blended for 2 minutes in the stomacher. Thereafter, 250 µL of the resuspended pellets were used for each sample. The DNA purity and concentration were measured with NanoDrop-1000 (NanoDrop Technologies, Wilmington, DE, USA). The DNA that was extracted was preserved at a temperature of −20 °C and as it served as a template DNA with primers in table 12 for the RT PCR.

Table 12: Identification of the various DEC virulence genes using primers

Dec	Patho-type	Gene	Orientation Primer Sequence	Reference
EPEC	*eaeA*	F	ATGCTTAGTGCTGGTTTAGG	(Wang *et al.*, 2002)
		R	GCCTTCATCATTTCGCTTTC	
EHEC	*Stx1*	F	CTGGATTTAATGTCGCATAGTG	(Lopez-saucedo *et al.*, 2003)
		R	AGAACGCCCACTGAGATCATC	
	Stx2	F	GGCACTGTCTGAAACTGCTCC	(Lopez-saucedo *et al*., 2003)
		R	TCGCCAGTTATCTGACATTCTG	
EIEC	*ipaH*	F	GTTCCTTGACCGCCTTTCCGATACCGTC	(Lopez-saucedo *et al.*, 2003)
		R	GCCGGTCAGCCACCCTCTGAGAGTAC	
EAEC	*eagg*	F	AGACTCTGGCGAAAGACTGTATC	(Omer *et al.*, 2010)
		R	ATGGCTGTCTAATAGATGAGAAC	
ETEC	*stIa*	F	TTTCCCCTCTTTTAGTCAGTCAA	(Guion *et al.*, 2008)
	stIb	F	TGCTAAACCAGTAGAGTCTTCAAAA	(Guion *et al.*, 2008)
	St	R	GCAGGATTACAACACAATTCACAGCAG	

3.4.2. Amplification of diarrhoeagenic *E. coli* virulence genes

All primers were synthesized Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa.

Primers were spinned at 4000 g for 30 seconds and reconstituted according to the manufacturer's instruction. Virulence genes characteristic of diarrhoeagenic *E. coli* were grouped into three different PCR sets. PCR set 1 involved amplification of *stx1 and stx2* (EHEC) and *ipaH* (EIEC) genes. PCR set 2 involved amplification of *eagg* (EAEC) and *eaeA* (EPEC) genes, and PCR set 3 involved amplification of *stIa* (ETEC) gene. PCR sets 1 and 2 were performed up to a final volume of 20 µL while 10 µL was the volume used for PCR set 3.

The reaction mixture for PCR set 1was made up of 10 µL of a 1x Luna® Universal qPCR (M3003G, New England BioLabs) Master Mix, 1.25 µL of each of the Forward (F) and Reverse (R) primers of *stx1* at a final concentration of 1.25 µM, 0.75 µL of each of F and R primer of *stx2* to 0.75 µM final concentration, 0.2 µL of each of R and F primer of *ipaH* at a final concentration of 0.2 µM, and 1.6 µL of (NF H_2O).

PCR set 2 comprised of 10 µL of Luna Master mix, 0.5 µL of F and R primers of *eaeA* at a concentration of 0.5 µM, 0.5 µL of F and R primers of *eagg* at a concentration of 0.5 µM and 5 µL of NF H_2O.

PCR set 3 contained a volume of 5 µL of Luna Master mix, 0.2 µL of the F primer of *stIa* at a concentration of 0.2 µM, 0.2 µL or the F primer of *stIb* at a concentration of 0.2 µM,

0.2 µL of the R primer of *st* at a concentration of 0.2 µM, 0.6 µL of NF H_2O, and 4µL of the DNA template.

Reactions were run on a Techne Prime Pro Real-Time PCR machine (Staffordshire, United Kingdom) using conditions in table 13. Reaction mixtures with no template DNA were used as Negative Controls (NTC) whiles positive controls were obtained from Food Research Institute (FRI) of the Council for Scientific and Industrial Research (CSIR), Ghana.

Table 13: Real-Time PCR conditions

Conditions	Temperature (°C)	Time(s)
Initial activation	98	50
Denaturation	95	10
Annealing	62	30
Final extension	72	20
Final incubation	4	

3.4.3 Agarose gel electrophoresis of genomic DNA

Agarose gel of 0.8 % (w/v) was prepared by weighing 0.32 g of agarose (molecular biology grade) into a conical flask containing 40 ml of 1×TAE buffer. The solution was heated in

the microwave oven for 2 minutes to melt the agarose and cooled to about 60 ^{o}C. The electrophoresis tray was sealed on both open ends with an autoclave tape. It was placed on the laboratory bench and checked with a balance to ensure a horizontal alignment. A comb of 15 teeth was inserted in the tray from one of the sealed ends. Ethidium bromide (4 µl) was added to the warm agarose solution and swirled gently to obtain a uniform mixture. The agarose solution was gently poured into the tray until the bottom of the tray was covered with the agarose solution. The agarose solution was allowed to gel for 30 minutes and the comb and autoclave tapes were removed gently. The gel was carefully mounted in the electrophoresis tank and the tank filled with 1×TAE buffer until the gel was covered with the buffer.

Aliquots of 10 µl DNA samples were transferred into newly labeled eppendorf tubes and 2 µl of the loading dye (bromophenol blue) was added. The mixture was slowly pipetted up and down until the content in the tubes were uniformly mixed. The 10 µl DNA prepared in each tube was loaded into the wells created by the comb and the order of loading noted. The first and the last wells were filled with the standard marker. The lid of the electrophoresis tank was put in place and the electrical leads were attached considering the position of the cathode and the anode. The gel was run for 45 minutes and subsequently viewed under UV transilluminator.

3.5. Physicochemical parameters

A portable pH meter (Mettler-Toledo GmbH, Greifensee Switzerland) was used to measure the pH of the samples. The pH meter was calibrated with distilled water. A 50 ml conical flask was filled with agitated sample and measurements were taken by dipping the

electrode into the sample. The pH of the *wagashi* was determined by measuring 5 g into 15 ml of buffered peptone water. The sample was blended for two minutes in the stomacher and the pH was measured. Each sample was measured three times.

3.6. Survival of *E. coli* O157:H7 isolated from *wagashi* to sub-lethal doses of low pH, salt and heat

3.6.1. *E. coli* acid-adaptation

Test tubes containing 9 ml of Tryptone Soy Broth (TSB; Oxoid CM0129, England) complemented with glucose of 1 % (Glucose 284504S, BDH laboratories, England) (TSB+G) were inoculated with two colonies of purified *E. coli* O157:H7 obtained from one *wagashi* sample and incubated for 24 h at 37 °C to obtain acid-adapted cells. The non acid-adapted *E. coli* O157:H7 cells were prepared by inoculating sterile test tubes containing 9 ml of TSB without glucose (TSB-G, pH 7.0) with 2 colonies of *E. coli* O157:H7 and incubating for 24h at 37°C (Buchanan, 1996). All treatments were in duplicates.

3.6.2. *E. coli* O157:H7 cultured on SMAC and acid stress in Mueller Hinton Broth

Aliquot of 1 ml of both the acid-adapted and non acid-adapted *E. coli* O157:H7 cultures at a concentration of 24×10^3 were aseptically transferred into test tubes containing 9 ml MHB (Oxoid CM0405, United Kingdom). The cultures were acidified with Lactic Acid (Saarchem, Wadeville, RSA) to a pH 3.5 and 4.0. Survival of *E. coli* O157:H7 was determined at times 0, 2, 4, 6 and 18 hours after incubation at 37 ^{o}C. One (1) ml of the suspension of both *E. coli* O157:H7 cultures were vigorously mixed using the vortex

(Stuart vortex mixer, SA7, United Kingdom) and diluted serially in 9 ml of 0.1 % sterile Peptone Water (PW, Oxoid CM0009, United Kingdom). One (1) ml of the samples were pour plated on Mueller Hinton agar (MHA) and incubated at 37 ℃ for 24 hours. Growth observed were counted on a colony counter (Stuart, colony counter SC6+, United Kingdom) and recorded.

3.6.3. *E. coli* O157:H7 salt stress in Mueller Hinton Broth

An aliquot of 1 ml of the acid-adapted *E. coli* O157:H7 as well as non-adapted *E. coli* O157:H7 cultures were aseptically transferred into new test tubes containing 9 ml each of MHB with salt concentrations 2 % and 3 % (v/v) NaCl (EC 231-598-3, Leuven Belgium). Both treatments were incubated for 3 days at 37 ºC. One (1) ml of the suspension of both the acid-adapted as well as the non-adapted *E. coli* O157:H7 cultures were vigorously mixed in 9 ml of 0.1 % sterile PW serial dilutions using the vortex. One (1) ml of the samples were pour plated with MHA and incubated at 37℃. The microbial load determined every 24 hours for 3 days.

3.6.4. *E. coli* O157:H7 heat stress using Mueller Hinton Broth

Heat stress was assessed by subjecting 1 ml of the 20 x 10^3 concentration of both the *E. coli* O157:H7 that has been adapted with acid as well as the *E. coli* O157:H7 that has not been adapted with acid cultures into 9 ml MHB at temperatures 50 ℃ and 55 ℃ for 30 minutes in a water bath. One (1) ml of the suspension of both the acid and non acid-adapted cultures were vigorously mixed using a vortex and diluted serially in 9 ml of 0.1 % sterile PW. One (1) ml of the samples were pour-plated on MHA and incubated at 37 ℃ for 24 hours. Growth observed were counted on a colony counter and recorded.

3.6.5. Survival of acid and non-acid adapted *E. coli* O157:H7 applying combined treatments of pH 4, 3 % salt at 50 ^{o}C in Mueller Hinton Broth

An aliquot of 1 ml of the sixth dilution of both the acid-adapted and non acid-adapted cultures were aseptically transferred into new test tubes containing 9 ml each of MHB with pH 4, 3 % NaCl (v/v) and incubated at a temperature of 50 ^{o}C for 2 h. *E. coli* O157:H7 survival in both cultures were determined on MHA using pour plating at 0, 30, 60, 90, and 120 minutes. Inoculated MHA plates were incubated at 37 ℃ for 24 h.

3.7. *E. coli* O157:H7 challenge test in *wagashi* processing

3.7.1 Preparation of Sodom apple extract

Ten grams (10 g) of fresh Sodom apple stem was washed and blended with 20 ml of fresh milk. The blend was sieved on a filter to obtain the extract which had a pH of 6.3.

3.7.2 Monitoring microbial populations in *wagashi* processing

A 100 ml fresh raw milk samples each were inoculated with 1 ml of 28 x 10^4 concentration of both the acid-adapted and the non acid-adapted *E. coli* O157:H7 separately. Both raw milk samples containing the inoculum were pasteurized at 72 ^{o}C for 15 s and cooled to 30 ^{o}C. Microbial quality was assessed immediately. Both inoculated samples were processed into *wagashi* by slowly heating the milk samples in beakers to a temperature of 50 ^{o}C. Six (6) ml of Sodom apple extract was added to each of the 100 ml milk samples. The samples were heated to 55^{o} C for 30 min to allow curdling of the milk. The samples were each poured into a basket to drain the whey.

3.7.3. Microbiological assessment

A 25 g of both freshly prepared *wagashi* and whey of both the acid-adapted and non acid-adapted samples were weighed into 225 ml of *E. coli* broth prepared according to the manufacturer's instruction. The samples were vigorously mixed in the stomacher and incubated for 3 hours at 37 ºC. One (1) ml of the *wagashi* samples and the whey of the *E. coli* O157:H7 that has been acid-adapted as well as *E. coli* O157:H7 that has not been adapted inoculated samples were serially diluted. One (1) ml of the cultures were pour plated after appropriated serial dilutions had been made immediately after pasteurization, after the milk was cooled at 30 ℃. Then immediately after the preparation of the *wagashi*, and after three days storage of *wagashi* at ambient temperature. Plates were incubated at 37 ºC for 24 h.

3.7.4 Innoculation of processed *wagashi*

Freshly prepared *wagashi* samples were heated in a water bath at 72 ℃ for 15 seconds and cooled to a temperature of 30 ℃. One (1) ml of both adapted as well as the non-adapted *E. coli* O157:H7 cells in the TSB were each inoculated separately unto two 5 g *wagashi* samples at a concentration of 18 x 10^3 to simulate post-processing contamination.

3.8 *E. coli* O157:H7 survival during storage of processed *wagashi*

Ten grams (10 g) of freshly prepared *wagashi* samples were heated in water bath at 72 ºC for 5 s and cooled to 30 ºC. The samples were inoculated with the 10x 10^4 of both the acid-adapted and non acid-adapted *E. coli* O157:H7 isolated from *wagashi* samples at an initial concentration of 4.1 log cfu/ml. Growth was observed for 5 days at ambient temperature

of 28 ℃. Growth of the *E. coli* O157:H7 was observed everyday by enriching in homogenized sample in 100 ml of *E. coli* broth. Samples were incubated for 3 h at 37 ℃. One (1) ml of the suspension of both acid-adapted and non acid-adapted cultures were diluted serially in a volume of 9 ml of 0.1 % sterile PW and vigorously mixed using the vortex. The samples (0.1 ml) were spread plated on MHA and incubated at 37 ℃ for 24 hours. Growth observed were counted on a colony counter and recorded.

3.9 Statistical analysis

Data were analyzed by reporting different microbial strains in a contingency table. Prevalence of *E. coli* and *E. coli* O157:H7 in dairy products were presented as percentages in frequency tables. One-way analysis of variance (ANOVA) was also used to compare means to determine significant effects of treatments (acid-adaptation, low pH, heat and salt concentration) on *E. coli* O157:H7 survival in Tryptose Soy Broth and during *wagashi* production and storage. All treatments were in duplicates. ANOVA was performed using STATGRAPHICS CENTURION XVI. The level of ANOVA was significant set at $P < 0.05$.

CHAPTER 4

4.0 RESULTS AND DISCUSSION

4.1 The prevalence of diarrhoeagenic *E. coli* from informal dairy products

Assessment of the prevalence of *E. coli* in informal fresh milk products in Greater Accra revealed that, more than two-thirds (70.6 %) of all the samples collected were contaminated with *E. coli* (table 14). This was slightly lower than that reported in Egypt from raw milk (76.4 %) and Karish cheese (74.5 %). Lower prevalence were reported in Zimbabwe (66.6 %) (Gran *et al*., 2003), Iran (26 %) (Adesiyun *et al.,* 1999; Farzan *et al.,* 2013), South Africa (23.3 %) (Lues *et al*., 2003), India (31.6 to 57 %) (Nanu *et al*., 2007); Singh and Gandhi, 2015) and Pakistan (50 to 65 %) (Soomro *et al.*, 2002). Higher prevalence of *E. coli* (96 to 97.7 %) has been reported in cheese in Brazil (Paneto *et al.*, 2007). The high *E. coli* prevalence observed in this research may be due to the unhygienic conditions under which the dairy products were processed.

From the individual samples, raw milk was the most contaminated. *E. coli* in raw milk is a natural occurrence since the cattle's large intestine is known to be a reservoir for *E. coli* (Zaheer *et al.*, 2017). Other sources of contamination could be poor hygienic practices on the farm during milking, use of unhygienic utensils for milking, storage and transport. The prevalence of *E. coli* in dairy implies a direct or indirect fecal contamination (Oliver *et al.,* 2009). This therefore, demands the practice of stringent hygienic practices during milk production and storage (Chaleshtori *et al.,* 2017).

Table 14 indicates that the prevalence of *E. coli* in boiled milk was 57.1 %. This was unusual as boiling is a much more stringent heat treatment compared to the required pasteurization treatment of milk. It was therefore expected that boiled milk would have undetectable levels of *E. coli*. The high prevalence of *E. coli* in boiled milk was most likely due to post-processing contamination as a result of poor hygienic practices including recontamination of milk with improperly sanitized utensils.

The prevalence of *E. coli* in the traditional dairy products is presented in table 14. *Brukina* recorded a higher prevalence (81.1 %) of *E. coli* than *nunu* (53.3 %). This higher prevalence of *E. coli* in *brukina* could be attributed to the addition of the steam cooked millet to the *nunu* during the preparation of the *brukina* from contaminated hands, exposed cooked and cooled millet or use of improperly sanitised utensils. The high prevalence of *E. coli* in these two local dairy products could be attributed to unhygienic practices employed by the processors or the use of inappropriate temperature control during transport and vending operations which could also support the growth of the *E. coli* cells which already exist in the product.

Table 14: Prevalence of *E. coli* in locally produced milk samples

Types of Dairy Products	Number of samples positive for *E. coli*		
	Number	**Positive samples**	**%**
Raw milk	4	4	100.0
Boiled milk	14	8	57.1
Wagashi	15	10	66.7
Nunu	15	8	53.3
Brukina	37	30	81.1
Total	**85**	**60**	**71**

The prevalence of *E. coli* in *wagashi*, a fresh coagulum (cheese) of raw milk, was 66.7 %. The prevalence of *E. coli* in the *wagashi* was relatively higher than the prevalence of *E. coli* reported in Ras cheese (21.7 %) (Ombarak *et al.*, 2016), and raw milk cheese (0 %) (Brooks *et al*., 2012). However, the prevalence of the *E. coli* was lower than the prevalence of the *E. coli* in the Karish cheese (74.5 %). The differences in the prevalence of *E. coli* in the various cheeses may be attributed to the variations in production processes of the cheese products and the microbiological quality of the raw materials.

Table 15 shows the prevalence of *E. coli* O157:H7 in the dairy samples after colonies of each sample were examined on SMAC agar. An overall prevalence of 25.9 % *E. coli* O157:H7 was detected in the tested dairy products. Also 20.0 % prevalence of *E. coli* O157:H7 was realized in *nunu*. This suggests that the consumption of raw milk possesses a high risk of fecal contamination than processed dairy products. The results from this study indicate the serious health risk that the public is likely to face by consuming these dairy products. The prevalence of *E. coli* O157:H7 in the indigenous milk products was observed to be higher in the raw milk samples (50.0 %) as compared to the other milk samples tested.

Table 15: Prevalence of *E. coli* O157:H7 using culture-based method

Types Of Dairy Products	Number of samples positive for *E. coli* O157:H7		
	Number	**Positive samples**	**%**
Raw milk	4	2	50.0
Boiled milk	14	3	21.4
Wagashi	15	6	40.0
Nunu	15	3	20.0
Brukina	37	8	21.6
Total	**85**	**22**	**26**

Lower prevalence of *E. coli* O157:H7 have been reported in various milk products. Ahmed and Shimamoto (2014) reported a prevalence of 3.6 % in milk; Alisarli *et al.* (2004) reported 3 % in milk, Aksu *et al*., (1999) reported 2 %) in milk. Conversely, a research by Sancak *et al*. (2015) on raw milk, *E. coli* O157:H7 was absent. The high prevalence of *E. coli* O157:H7 could be attributed to either direct or indirect fecal contamination of the milk during milking or post processing contamination through unhygienic practices such as not

using antibacterial soap and potable water for washing hands and proper cleaning and sanitization practices for utensils before and after milking. Ahmed and Shimamoto (2014) who reported 1.6 % STEC contamination of fresh beef, suggested that *E. coli* O157:H7 could result from cross-contamination with unhygienic utensils. The presence of *E. coli* O157:H7 may also be due to unhygienic practices of cross contamination with the use of same measuring cups for raw and processed dairy. Although consumption of raw milk in Ghana is low, dairying households in Ghana have attested to consuming raw milk (Arthur, 2016) and thus there is a food safety risk if the raw milk consumed contain significant levels of *E. coli* O157:H7. Highly virulent levels of 10-100 cells of *E. coli* O157:H7 can cause disease (Van Giau *et al.*, 2016) therefore its prevalence in ready-to-eat dairy products is a public health concern.

The prevalence of the diarrhoeagenic *E. coli* was determined using the real-time PCR. Tables 16 and 17 present a summary of diarrhoeagenic *E. coli* prevalence in the informal dairy products.

Table 16: Number of samples positive for the various DEC virulence genes out of the 85 sampled local dairy products

Sample type	Number of samples positive for *E. coli* virulence genes					
	EHEC		EIEC	EPEC	EAEC	ETEC
	stx1	*stx2*	*ipaH*	*eaeA*	*eagg*	*Stl*
Raw milk (n=5)	1	1	1	1	1	0
Boiled milk (n=0)	0	0	0	0	0	0
***Brukina* (n=10)**	4	2	2	0	1	2
***Nunu* (n=7)**	3	1	0	1	1	1
***Wagashi* (n=3)**	2	0	0	0	1	0
Total	10	4	3	2	4	3

Out of the six diarrhoeagenic *E. coli* studied, *stx1* and *stx2* gene (EHEC) were most prevalent in all the five different dairy samples but boiled milk which indicate that it is the most prevalent among all the other genes. This corresponds with the prevalence of *E. coli* O157:H7 in local dairy products. The second most prevalent gene identified was *eagg* (EAEC) which was found in four (raw milk, *brukina, nunu and wagashi*) different dairy samples. The dairy product that did not contain this two pathogenic *E. coli* was raw milk. The least prevalent gene identified in the study were *eaeA* (EPEC) and *eagg* (EAEC) which were identified in three (raw milk, boiled milk and *brukina*) and four (boiled milk, *brukina,nunu, and wagashi*) different types of dairy products. Some other samples that

contaminated more than one type of diarrhoeagenic *E. coli* were also identified in table 17 and 18.

Brukina samples were identified to be most contaminated and with high prevalence of two or more of almost all the six different diarrhoeagenic *E. coli.* This suggests that *brukina* is very favorable and a good medium for the growth of the microorganism. Some producers add the supernatant of soaked corn or the supernatant of overnight soaked corn dough to mix the milk to serve as a starter culture to obtain the sour aftertaste of the *brukina*. The addition of the supernatant of the corn or corn dough could introduce contaminants including diarrhoeagenic *E. coli* into the *brukina* beverage. The real-time PCR technique identified 7 *brukina* samples, each of which tested positive for two different diarrhoeagenic *E. coli.* One *brukina* sample had two different strains of EHEC identified in fig. 12, others had the following combinations in the same sample: (EHEC) ie. *stx1 and stx2*, EHEC+EIEC, EHEC+EPEC, EHEC+ETEC, EAEC+ETEC, EAEC+EPEC, and EPEC+ETEC. Two *brukina* samples each tested positive for three combinations of diarrhoeagenic *E. coli*. The combinations were EHEC (*stx1*&*stx2*) + EIEC and EHEC+EPEC+ETEC.

Table 17: Number of samples positive for two or more of the various diarrhoeagenic *E. coli* virulence genes out of the 85 sampled local dairy products

Samples	*Stx1+ Stx2*	*Stx1+ ipaH*	*Stx1+ eaeA*	*Stx1+ StI*	*Stx2+ ipaH*	*Stx2 +eaeA*	*eagg+ StI*	*eagg+ eaeA*	*eaeA + StI*	*Stx1+ Stx2 +ipaH*	*Stx1+ eaeA+ StI*	n
Raw milk	0	0	0	1	0	0	1	0	0	0	0	2
Boiled milk	0	0	0	0	0	0	0	0	0	0	0	0
Brukina	2	1	2	1	1	2	0	0	1	1	1	12
Nunu	0	1	0	0	0	0	0	0	1	0	0	2
Wagashi	0	0	1	0	1	0	0	1	0	0	0	3
Total	2	2	3	2	2	2	1	1	2	1	1	19

stx1=EHEC, *stx2*=EHEC, *ipaH*=EIEC, *eagg*=EAEC, *stI*=ETEC and *eaeA*=EPEC n=Number of samples containing more than one diarrhoeagenic *E. coli* in each dairy product.

Table 18: Summary of samples containing more than one diarrhoeagenic *E. coli* virulence gene identified in local dairy products

Dairy product	N	Number of samples containing more than one DEC)	DECs identified
Raw milk	4	2	EHEC and ETEC, EAEC and ETEC
Boiled milk	14	-	-
Brukina	37	12	EHEC and EIEC, EHEC and EPEC, EHEC and ETEC, EHEC and EIEC, EPEC and ETEC, EHEC, EPEC and ETEC
Nunu	15	2	EHEC and EIEC, EAEC and ETEC
Wagashi	15	3	EHEC and EPEC, EHEC and EIEC, EPEC and EAEC
Total	85	19	

stx1=EHEC, *stx2*=EHEC, *ipaH*=EIEC, *eagg*=EAEC, *stI*=ETEC and *eaeA*=EPEC

Identification of samples positive for diarrhoeagenic *E. coli* by agarose gel electrophoresis

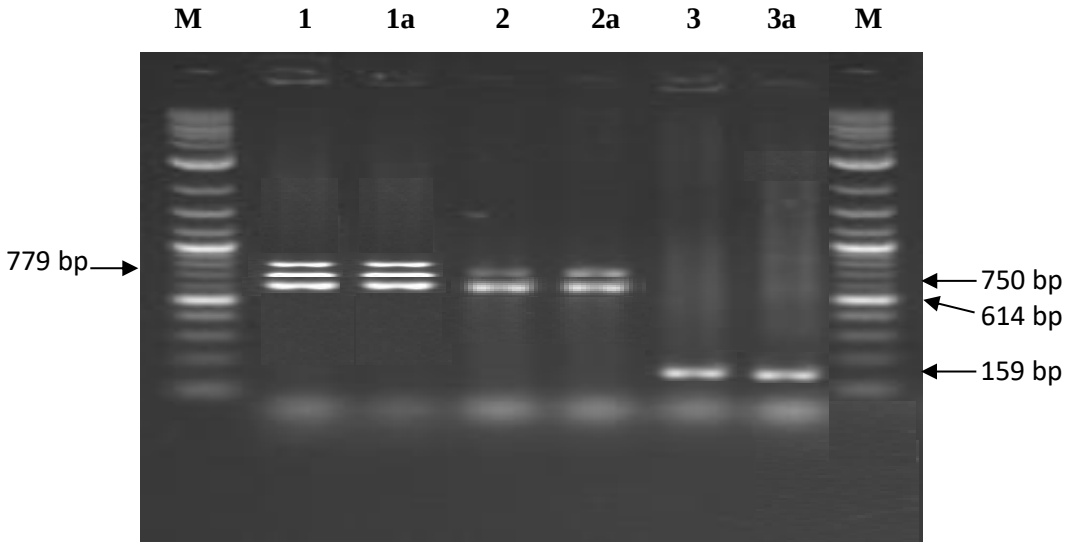


Fig. 12: PCR amplification products of pathogenic *E. coli* using species specific primers. Lanes: 1, 1a = *Stx1*F/R primer pair (Enterohaemorrhagic *E. coli*), *Stx2*F/R primer pair (Enterohaemorrhagic *E. coli*) and IPAHF/R primer pair (Enteroinvasive *E. coli*) in multiplex; Lanes: 2, 2a = *eaeA*F/R primer pair (Enteropathogenic *E. coli*) and *eagg*F/R primer pair (Enteroaggregasive *E. coli*) in multiplex; Lanes: 3, 3a = *StI*F/R primer pair (Enterotoxigenic *E. coli*); M = Molecular ladder (100 -10,000 bp).

4.2 Responses of acid-adapted and non-acid adapted *E. coli* O157:H7 to sublethal heat, low pH and salt stresses in Mueller Hinton broth

4.2.1 Effect of pH on both acid-adapted and non acid-adapted *E. coli* O157:H7

E. coli O157:H7 prefer neutral pH, but it can survive at pH ranging 4.3 to 10 (Fang, 2013). Studies have shown that *E. coli* O157:H7 employs pH homeostatic systems to neutralize acidic effects on the cell under its normal growth pH range (Kaur and Asea, 2017). Under mild acidic conditions, *E. coli* O157:H7 can become adapted and resist subsequent lethal acidic conditions through specific mechanisms such as the arginine, glutamate and the

lysine decarboxylase systems (Parry-Hanson *et al.,* 2010). Acid adaptation has been shown to induce cross-protection against other non-acid stresses in *E. coli* O157:H7. Sigma S (RpoS) is what intervenes cross protection of the adapted *E. coli* O157:H7.

In this study, the *E. coli* O157:H7 cells that has been adapted with acid are subjected to acidic pH of 4.0 in Mueller Hinton Broth survived much better, showing slight increase in cell concentration after 18 hours of storage at ambient temperature compared to the non acid-adapted control under same conditions (fig. 13). At the end of the 18 hours, acid-adapted *E. coli* O157:h7 had an average of 5.975 log cfu/ml which was significantly higher ($p=0.0012$) than 1.295 log cfu/ml recorded for the non acid-adapted *E. coli* O157:H7 cells in the media of pH 4. Survival of non-acid adapted *E. coli* at pH4 also suggest inherent bacterial resistance to low pH. *E. coli* O157:H7 adapted cell growth in a media at pH 4 differ significantly from one duration to another. The non acid-adapted *E. coli* O157:H7 cells that were not acid-adapted observed a significant decline of ($p=0.0052$) from one duration to the other. As indicated in figure 5, a similar observation was made for both cells of *E. coli* O157:H7 that has been adapted with acid and the ones that has not been adapted with acid in media of pH 3.5 in fig. 14. With respect to the acid-adapted *E. coli* O157:H7, the exposure of the acid-adapted *E. coli* O157:H7 to the sublethal acidic concentration might have enhanced their survival for a longer period as compared to the nonexposure of the *E. coli* O157:H7 to mild acidic conditions. The exposure of the acid-adapted *E. coli* O157:H7 in the sublethal acid concentrated media might have increased the percentage of cyclopropane (cy 17:O and cy 19:0) while the monounsaturated fatty acids (16:1w7c and 18:1w7c) decreased significantly in the cell membrane (Brown *et al.,* 1997). It was explained by Brown *et al.* (1997) that the change in the lipids within the membrane is

significant in ensuring both membrane integrity and functionality in the presence of external perturbation.

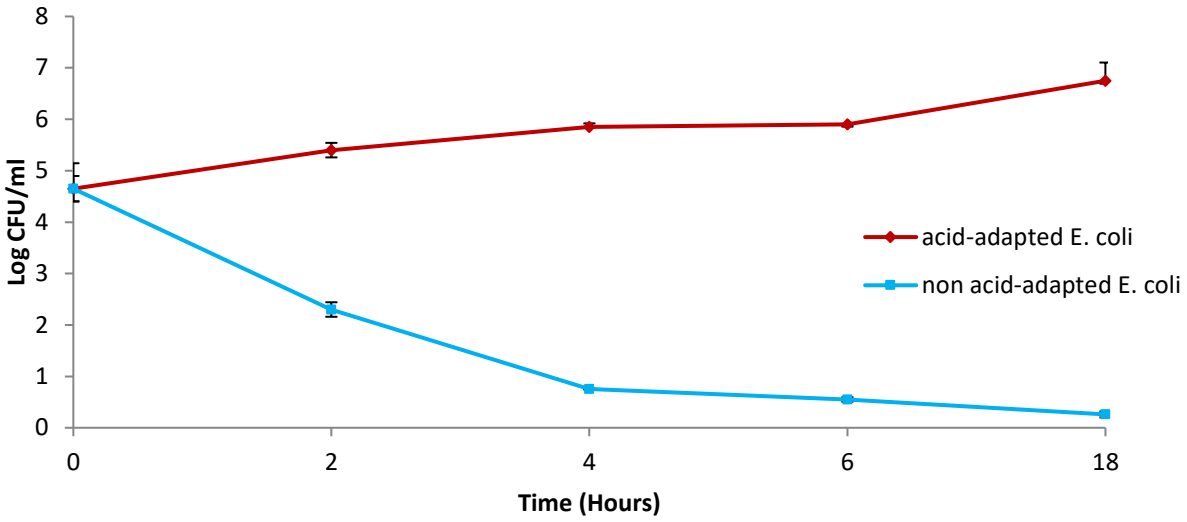


Figure 13: *E. coli* O157:H7 survival of both adapted and non adapted cultures in Mueller Hinton Broth at pH 4

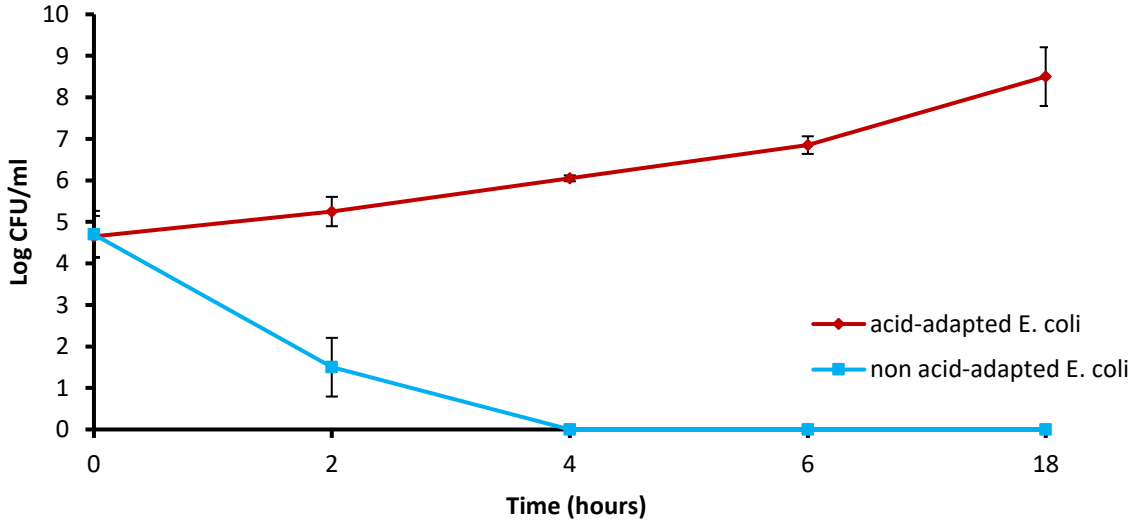


Figure 14: Survival of acid-adapted and non acid-adapted *E. coli* O157:H7 in Mueller Hinton Broth at pH 3.5

The findings in this study are consistent with the findings of Ryu and Beuchat (1998) and Sainz *et al.* (2005) who indicated that acid-adapted *E. coli* had better growth and survival in acidic foods. However, Brown *et al.* (1997) reported a significant decrease in the concentration of acid-adapted *E. coli* O157:H7, although the reduction was gradual compared to the non acid-adapted *E. coli* O157:H7 which decreased rapidly during a 4 hour challenge test at pH 3.5. A similar observation was made by Zhang *et al.* (2017) who reported that acid-adapted *E. coli* O157:H7 treated with acetic acid and lactic acid had less growth as compared to *E. coli* O157:H7 cells that had not been acid-adapted. Conversely, Parry-Hanson *et al.* (2009b) reported low growth for the adapted *E. coli* O157:H7 cells associated to *E. coli* O157:H7 cells without acid adaptation in fresh goat milk (pH 6.9). This finding was consistent with the findings of Hsin-Yi and Chou (2001) who observed a decline in growth of the adapted cells of *E. coli* O157:H7 as compared with no *E. coli* O157:H7 adapted cells in diluted fermented milk known as Yakult (pH 3.9) as well as yoghurt (pH 3.9) at a temperature of 7 ℃ for a period of 6 days. Parry-Hanson *et al.* (2009b) explained that even though acid-adaptation triggers protection mechanism to improve lethal pH values persistence, of the process of adaptation might have augmented the of *E. coli* O157:H7 cells sensitivity to stress experienced in the goat milk. It was further explained by the researchers that the upkeep of acid-adaptation is a process that involves energy, and that there may be re-adaption of the cells to the pH of the milk which is a process that could be stressful and also influence growth.

The effect of low pH(3.5) on survival of both cultures of *E. coli* O157:H7 that has been adapted with acid and that culture that has not been adapted is presented in fig. 14. The acid-adapted *E. coli* O157:H7 had a significant increase ($p=0.0018$) in growth by 4.7 log

cfu/ml to a value of 8.5 log cfu/ml after 18 hours. However, the concentration of non acid-adapted *E. coli* O157:H7 observed a decline of 4.7 log cfu/ml at 0 h to non-detectable levels after 4 hours of incubation. The results indicated that the low pH of 3.5 observed significant lethal result (p=0.0003) encounted by non acid-adapted *E. coli* O157:H7. On the other hand, the acid-adapted *E. coli* O157:H7 was able to withstand the lethality of the low acidic pH. The lethal effect of the low pH in the non acid-adapted *E. coli* O157:H7 could be attributed to denaturation of the proteins responsible for the metabolic activity of the organism, resulting in their death.

Also, an average of 6.662 log cfu/ml growth was recorded for acid-adapted *E. coli* O157:H7 cells which was significantly higher (p=0.0018) than 0.375 logcfu/ml recorded for the *E. coli* O157:H7 cells that was not acid-adapted (p=0.0003) in minimal media at pH 3.5. The growth of the cells of *E. coli* O157:H7 that has been adapted with acid in Mueller Hinton broth differ significantly from one duration to the other whiles the non acid-adapted *E. coli* O157:H7 cells declined significantly from one duration to the other.

4.2.2 Effect of temperature on both acid-adapted and non acid-adapted *E. coli* O157:H7

The use of heat treatment in the processing of food like milk is very common. Processing the food beyond the temperature range required for the growth of the target microorganisms is very vital in ensuring the preservation and extension of the shelf life of food. *E. coli* O157:H7 has a temperature growth range of 30-42 ℃, it is therefore expected that at the temperatures 50 and 55 ℃ employed for this work in fig. 15. will have some degree of lethality on the *E. coli* O157:H7 cells.

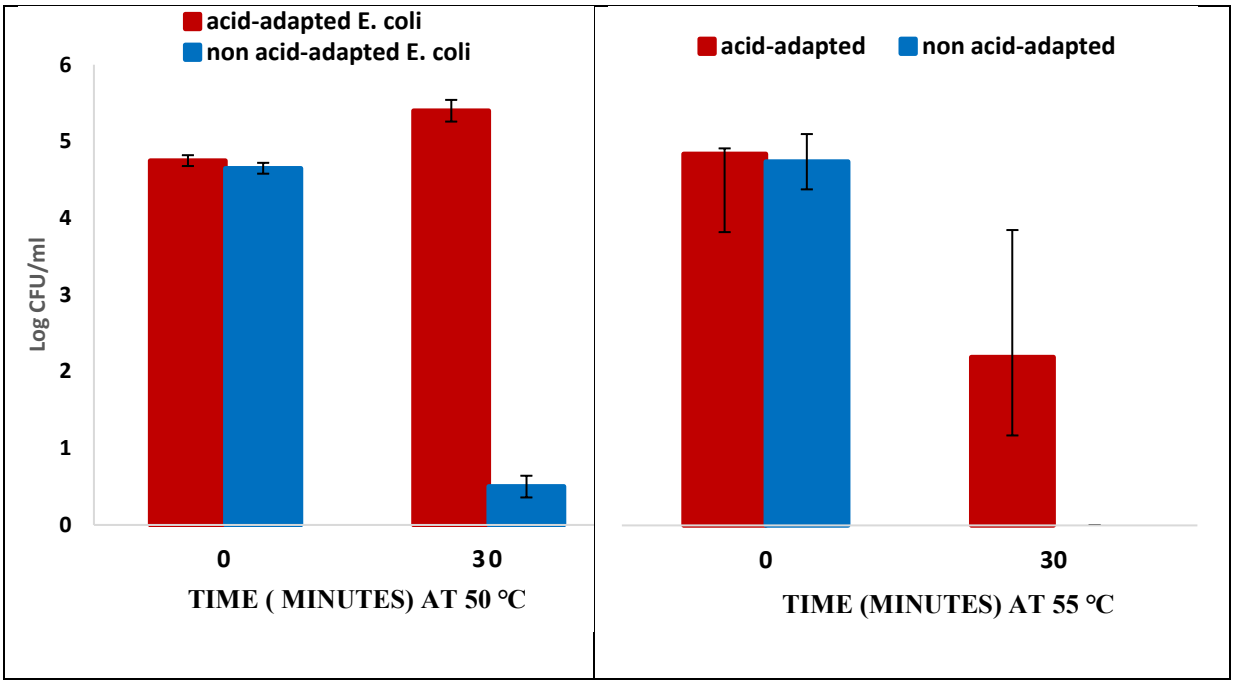


Figure 15: Survival of both adapted and non-adapted cells of *E. coli* O157:H7 at temperatures of 50 ℃ and 55 ℃ in Mueller Hinton Broth

A temperature of 50 ℃ significantly reduced (p=0.0007) the non acid-adapted *E. coli* O157:H7 but the acid-adapted *E. coli* O157:H7 showed marginal but significant growth (p=0.0283) as observed in figure 15.

At a temperature of 55 ℃, the reduction in both the acid-adapted and non acid-adapted *E. coli* O157:H7 cells were observed as depicted figure 15. This reduction was not significant for acid-adapted cells (p =0.1524), but the non acid-adapted *E. coli* O157:H7 cells were reduced beyond dection (p= 0.0029). Acid-adaptation of *E. coli* cells induces cross-protection through Sigma S regulated Oxidative system to confer protection to other unrelated stresses including heat stress (Parry-Hanson *et al.*, 2010). This is done by co-activation of protective enzymes such as chaperonins by Sigma S (Kolbus *et al.*, 2003).

The resistance of acid-adapted *E. coli* O157:H7 cells to heat at 55 ˚C for 30 minutes, could be attributed to the cross-protection induced by acid-stress response (Parry-Hanson *et al.*, 2009a). The findings in this work is similar to the observation reported by Velliou *et al.*, (2011) who observed an increase in the resistance of acid treated or acid-adapted *E. coli* K12 cells to heat.

4.2.3 Effect of salt on both acid-adapted and non acid-adapted *E. coli* O157:H7

An increase in the osmolarity of the environment can be very damaging to the growth and survival of *E. coli* O157:H7 since it prompts a rapid efflux of water from the cell, resulting in a decreased turgor (Lucht and Bremer, 1994). This implies that the *E. coli* must therefore promptly sense changes in the external osmolarity and instigate appropriate stress responses (Cheng *et al*., 2003). According to Lucht and Bremer (1994), the loss of turgor triggers a rapid influx of K^+ ions into the cell via specific transporters and the concomitant synthesis of glutamate. However, at high osmolarity, K^+- glutamate is insufficient to ensure cell growth and the *E. coli*, therefore, replace the accumulated K^+ ions with compounds such as trehalose and amino acids such as proline and methyl-amines such as glycine that are less harmful to the cell's physiology (Lucht and Bremer, 1994). Results from this study suggests that acid-adaptation may have contributed to the survival of adap-adapted *E. coli* O157:H7 to 2 % and 3 % salt compared to non acid-adapted controls as observed in fig. 16 and 17 respectively.

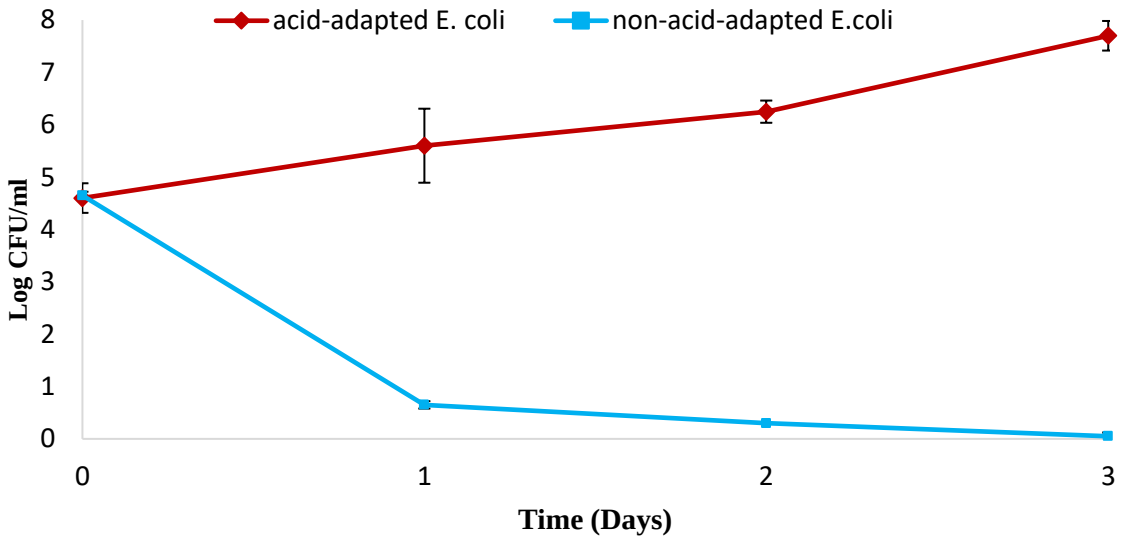


Figure 16: Survival of acid-adapted and non acid-adapted *E. coli* O157:H7 in Mueller Hinton Broth of 2 % concentration of salt at ambient temperature

The acid-adapted *E. coli* O157:H7 cells showed a gradual increase in cell concentration from 4.60 cfu/ml to 7.70 log cfu/ml while the cell count of the non acid-adapted *E. coli* O157:H7 cells declined drastically from 4.65 log cfu/ml to 0.05 log cfu/ml at the end of a 3 days challenge test within the media of 2 % salt concentration. As indicated in fig. 18, a similar trend was observed for the acid-adapted and non acid-adapted *E. coli* O157:H7 cells. However, a drastic decline of the cells was observed for the non acid-adapted *E. coli* O157:H7 cells after 3 hours in 3 % salt concentrated media. The lethal effect of the increase in salt concentration to 2 % and 3 % in the non acid-adapted *E. coli* O157:H7 cells could be due to increasing osmolarity of the media causing efflux of water from the cells, resulting in decreases turgor (Lucht and Bremer, 1994).

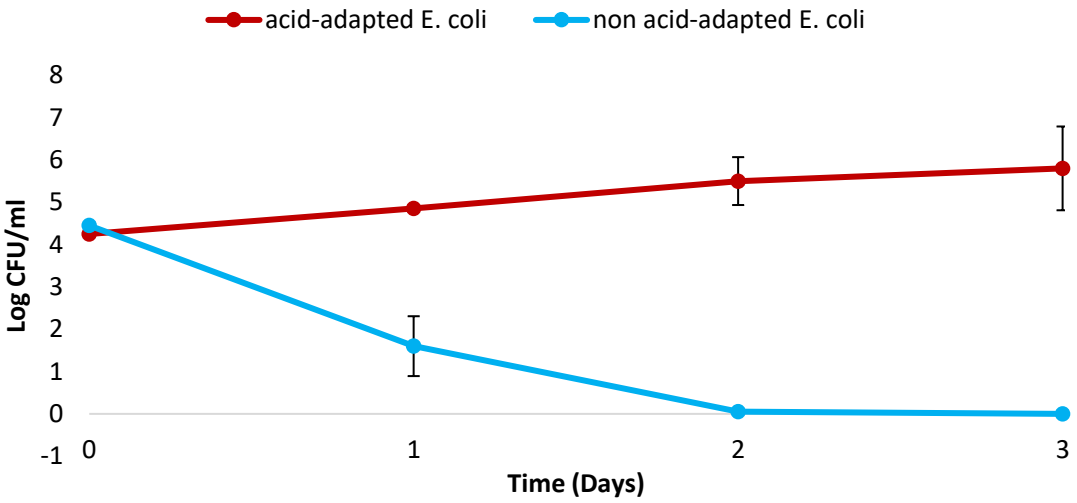


Figure 17: Survival of *E. coli* O157:H7 in Mueller Hinton Broth of 3 % salt concentration at ambient temperature

Form the 2 % salt concentration challenge test, the average cell count of the acid-adapted *E. coli* O157:H7 cells was 6.52 log cfu/ml which is significantly higher than the average cell count of the non acid-adapted *E. coli* O157:H7 cells which recorded 0.33 log cfu/ml for the three days.

The cell count of the acid-adapted *E. coli* O157:H7 increased significantly (p=0.0078) as the duration increased in the media containing 2 % salt while the cell count of the non acid-adapted *E. coli* O157:H7 decreased significantly (p=0.00) to non detectable levels on day 3. For the 3 day period of the 3 % salt concentration challenge test, the average cell count of the acid-adapted *E. coli* O157:H7 cells was 5.38 log cfu/ml which was significantly higher than the average cell count of 0.55 log cfu/ml recorded for the non acid-adapted *E. coli* O157:H7 cells.

The cell count of the acid-adapted *E. coli* O157:H7 on day 3 was not significantly different from that of day 0 (p =0.1636). However, the cell count of the non acid-adapted *E. coli*

O157:H7 decreased significantly (p =0.0007) from day 0 to day 3. This implies that increased in the salt concentration of the media had a lethal effect of the non acid-adapted *E. coli* O157:H7 cells whiles no significant effect on the acid-adapted *E. coli* O157:H7 cells was observed.

4.2.4 Effect of combined treatments on both acid-adapted and non acid-adapted *E. coli* O157:H7 (pH 4, 3 % salt concentration and temperature 50 ℃)

Generally, the use of two or more inhibitory agents, like acid and salt is more effective against microbes than when one inhibitory agent is used (Bae and Lee 2017). This concept is known as the hurdle concept and has been recommended to use in food technology applications especially when minimal processing is employed. In this study, the combined treatment of lactic acid treatment at pH 4, salt treatment at 3 %, and heat treatment at 50 °C inhibited both acid-adapted and non-acid adapted *E. coli* O157:H7 albeit at different rates (fig. 18). The longer survival of the acid-adapted *E. coli* O157:H7 cells as compared to the non acid-adapted *E. coli* O157:H7 cells could be attributed to the cross-protection induced by the exposure of the cells to sublethal acidic environment prior to the exposure to the combined treatments.

This finding is similar to the findings of Bae and Lee (2017) who observed a synergistic inhibitory effect on *E. coli* O157:H7 in phosphate buffer, buffered peptone water and peptone water. However, according to Yoon *et al.*, (2016) the addition of salt increases the acid resistance of *E. coli* O157:H7 possibly by increasing its acid tolerance response and consequently preventing the acidification of its cytoplasm by organic acids. A similar

observation of the antagonistic effect of acetic acid and salt was observed by Bae and Lee (2017) in Lauria Bertani (LB) broth.

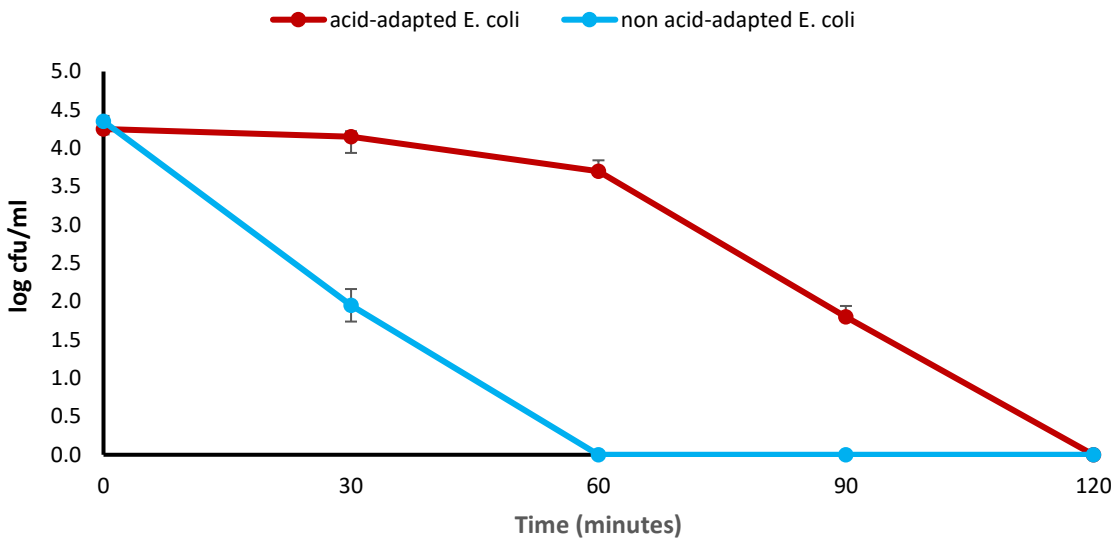


Figure 18: Survival of acid-adapted and non acid-adapted *E. coli* O157:H7 to combined treatment of temperature 50 °C, salt 3 % and pH 4 in Mueller Hinton Broth

Combined treatments significantly reduced cell numbers of both acid-adapted and non acid-adapted *E. coli* O157:H7 cells. This suggests that, application of hurdle technology could be effective to reducing *E. coli* O157:H7 cells that become adapted by pre-exposure to mild sublethal environmental conditions during processing or storage of milk especially in *wagashi* processing and storage.

4.3 Survival of acid-adapted and non acid-adapted *E. coli* O157:H7 in *wagashi* processing and storage

4.3.1 Challenge Test

As presented in figure 19, pasteurized milk did not have any detectable levels of *E. coli* O157:H7. This indicates that the temperature and time indicated by Hayes *et al.,* (2001) for pasteurization of milk was adequate to injure *E. coli* O157:H7 cells sufficiently to prevent growth in the milk sample. It was also observed that there was no microorganism detected during the time of curdling and cooling of *wagashi* cheese.

After the temperature of the sample had been cooled to room temperature, the *wagashi* samples were stored in the whey at ambient temperature (27-28℃) in a sealed polyethene sheet for three days. The whey and *wagashi* samples were assessed for *E. coli* O157:H7 survival daily for three days.

E. coli O157:H7 cell concentration increased gradually over time throughout the storage period of 3 days in both the *wagashi* and the whey. Generally, in the three days of the study, there was a significant growth of the non acid-adapted *E. coli* O157:H7 cells from 0.4 log cfu/ml from the time of cooling to 3.1 log cfu/ml at the end of the third day. The significant growth of the non acid-adapted *E. coli* cell could be that conditions of both the *wagashi* and the whey were very favorable for it. For the acid-adapted *E. coli* O157:H7, the growth of the cells was from 0.2 log cfu/ml to 2.9 log cfu/ml. The slow growth of the acid-adapted *E. coli* O157:H7 could be that the cells were readapting to its new environment after encountering a sublethal acid stress. *E. coli* O157:H7 observed on day one and afterwards could be as a result of residual persistent *E. coli* O157:H7 cells that were perhaps injured

so did not grow on SMAC plates. These cells may have repaired injuries caused by heat treatment to eventually grow during *wagashi* storage.

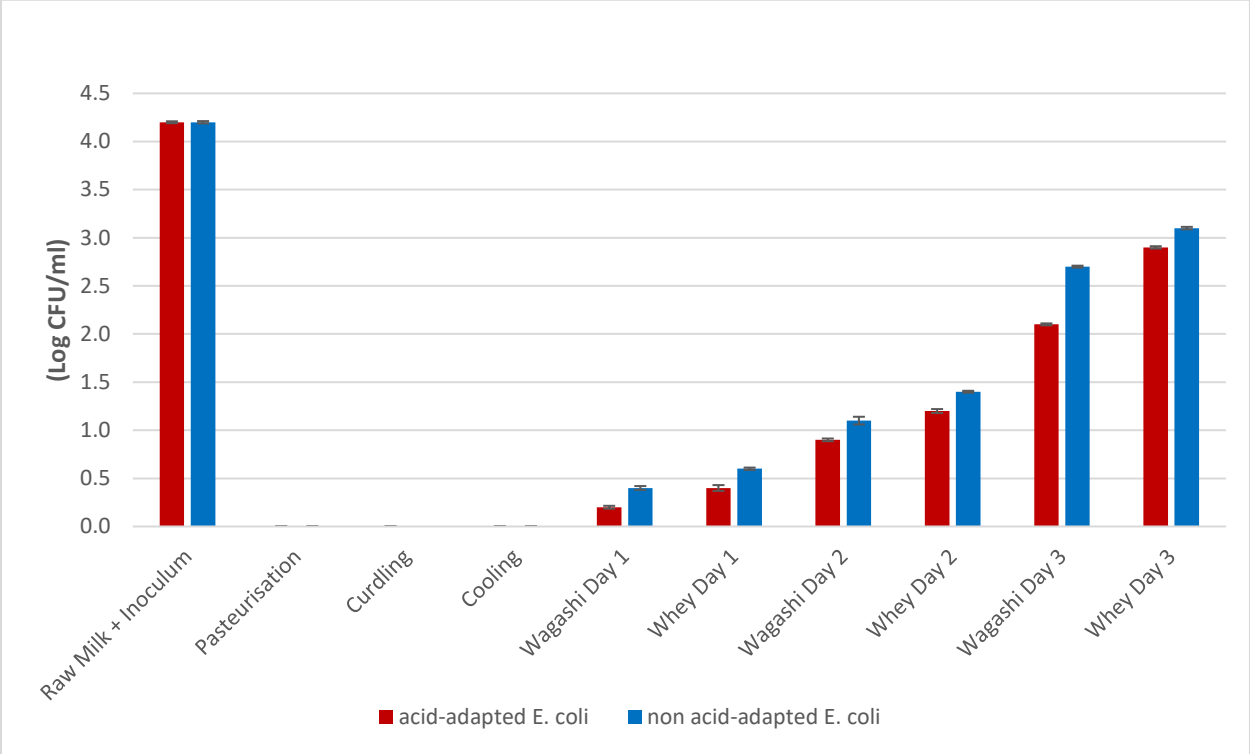


Figure 19: Microbial survival of acid-adapted and non acid-adapted *E. coli* O157:H7 during *wagashi* processing

4.3.2 Post processing contamination

From figure 20, post processing contamination was simulated to assess the growth of acid-adapted and non acid-adapted *E. coli* O157:H7. At this point the *wagashi* samples in the whey were pasteurized at 72 ℃ for 15 seconds, microbial analysis was conducted and there was no *E. coli* O157:H7 detected at this point.

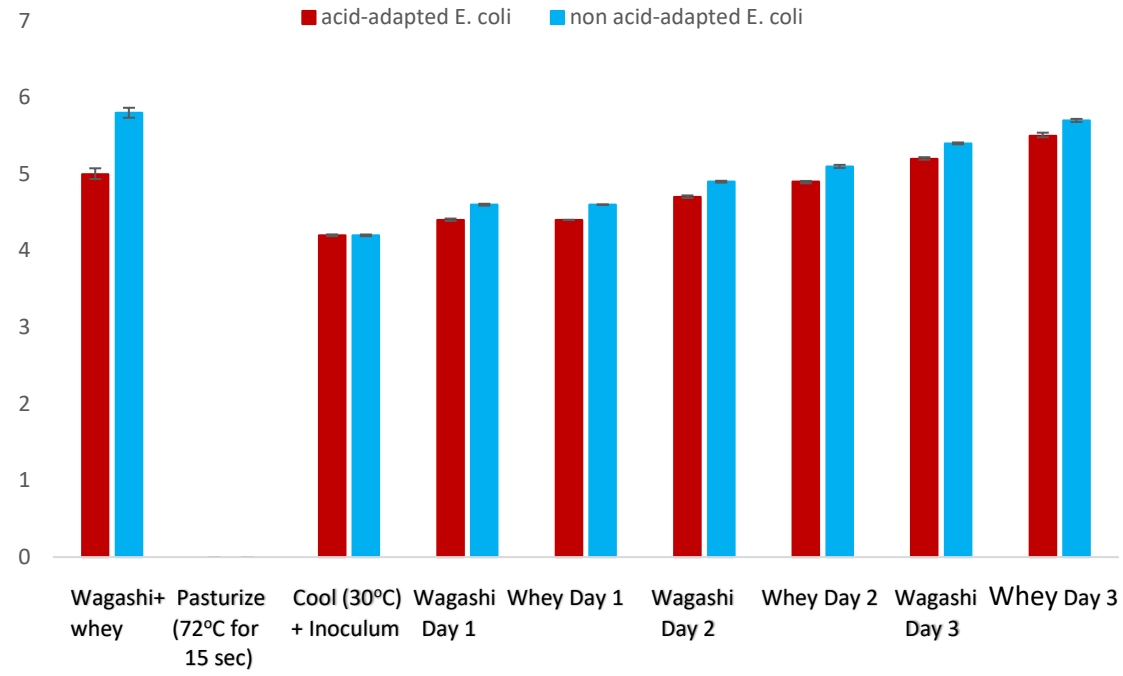


Figure 20: Simulating Post Processing Contamination of acid-adapted and non acid-adapted *E. coli* O157:H7 in *wagashi* and whey samples

After inoculation of the *wagashi* with 0.2 log cfu/ml concentration of the inoculum, growth commenced for both the acid-adapted and the non acid-adapted *E. coli* O157:H7 though the *wagashi* had a lower pH of 6.60. However, it was observed that the non acid-adapted *E. coli* O157:H7 cells grew better in cell concentration as compared to the acid-adapted *E. coli* O157:H7 cells but, growth in both the acid-adapted and the non acid-adapted *E. coli* O157:H7 cells was not statistically significant. This was also observed in a research done by Parry-Hanson *et al.*, (2009b). It was explained that, though acid-adaptation triggers protection mechanism to enhance survival, the process might have increased sensitivity of *E. coli* O157:H7 cells because maintenance of acid-adaptation is an energy driven process thus the acid-adapted *E. coli* O157:H7 cells may have re-adapted to the normal conditions in the environment. Growth was however observed but not as in the non acid-adapted cells.

4.3.2 Shelf life study

It was observed in fig. 21 during the shelf life study that, there was marginal growth both in the acid-adapted and the non-acid adapted *E. coli* O157:H7 cells for the 5-day challenge test in the *wagashi*. For the 5-day challenge test, the average cell count for the acid-adapted *E. coli* O157:H7 was 4.79 log cfu/ml which was significantly different (p = 0.0005) from 5.12 log cfu/ml which was recorded for the non acid-adapted *E. coli* O157:H7 cells.

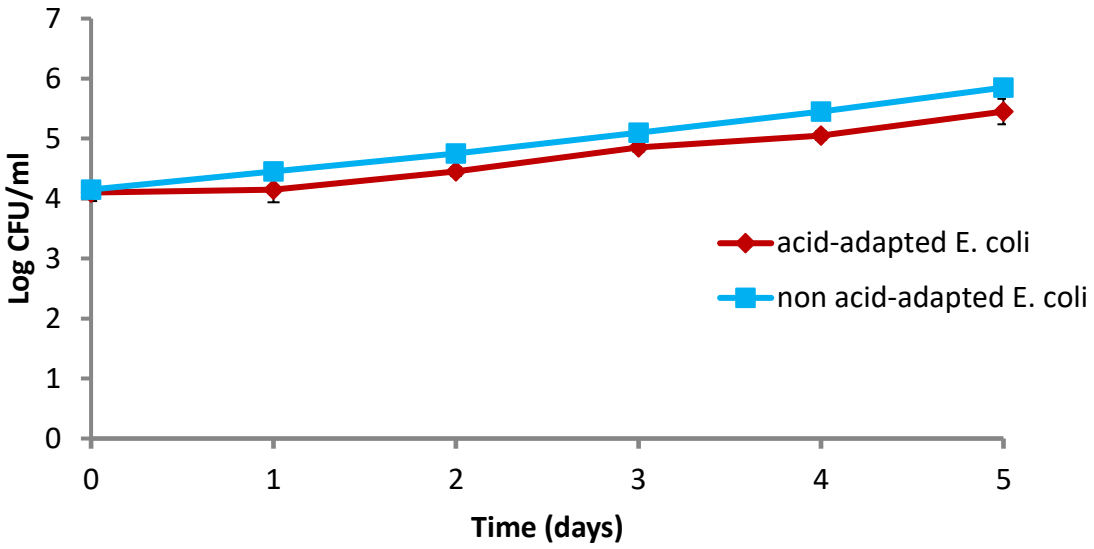


Figure 21: Survival curve of acid-adapted and non-adapted *E. coli* O157:H7 in *wagashi* sample kept in 1 % salted whey

The cell concentration of the acid-adapted *E. coli* O157:H7 was not significantly different at the various time points. Howevere, preexposure of the acid-adapted *E. coli* O157:H7 to sublethal treatment may have helped it to easily readapt to its new environment from day one to day five thereby increasing its cell concentration from 4.15 log cfu/ml to 5.45 log cfu/ml. There was also no signifucant growth observed in the first two days of the non acid-

adapted *E. coli* O157:H7. This may be because the cells of the non acid-adapted *E. coli* O157:H7 may have become sensitive to their new environment. Addition of the salt might have increased the osmolarity of the its cells thereby causing an efflux of water from the cells, leading to decreased turgor pressure in the cells of the non acid-adapted *E. coli* O157:H7. This process is energy driven, which may have caused the slow rate of growth of the non acid-adapted *E. coli* O157:H7 cells in the first two days. Also, there was no significant growth observed from day three to day five of the non acid-adapted *E. coli* O157:H7 but there was an increase in the cell concentration of the non acid-adapted *E. coli* O157:H7 cells from 4.15 log cfu/ml to 5.85 log cfu/ml at the end of the fifth day. The reason may be that the cells of the non acid-adapted *E. coli* O157:H7 may have adapted to its new enviromment therefore, normal growth of the cells of the non acid-adapted *E. coli* O157:H7 resumed.

CHAPTER 5

5.0 CONCLUSION AND RECOMMENDATION

5.1 Conclusion

This work presents the prevalence of *E. coli* and diarrhoeagenic *E. coli* in informal dairy products sold in some parts of Accra. Out of the 85 milk samples, 60 (70.6 %) tested positive for *E. coli*. Using multiplex RT PCR assay, 45 (52.9 %) tested positive for DEC and out of that, 19 samples (42.2 %) recorded more than one DECs. This makes most of the samples unsafe for consumption due to the exposure of consumers to these significant pathogens in the ready-to-eat dairy products.

At lethal processing conditions acid-adapted *E. coli* O157:H7 did not only survive but also exhibited significant growth in minimal media. However, acid-adapted *E. coli* O157:H7 was sensitive upon the application of more than one processing treatments. This suggests that when acid-adaptation occurs as a result of exposure of *E. coli* O157:H7 cells to mild acid environmental conditions, the application of appropriate hurdle technologies alongside good hygienic practices could potentially control their survival and growth in dairy.

It was observed that contamination of *wagashi* was predominantly either during processing or after processing. Boiling of *wagashi* from the study was observed as the most effective way of minimizing *E. coli* O157:H7.

5.2 Recommendation

Hygienic SOPs should be developed for processors and retailer of informal dairy products. It is important that the relevant stakeholders are trained on safe food handling, storage and the use of hygienic SOPs to improve hygienic handling of dairy products. Milk pasteurization and the appropriate use of hurdle technology in the processing of traditional dairy products are important to control enteropathogens like diarrhoeagenic *E. coli* that occur in raw milk. For more specialized operations, a HACCP system is recommended to appropriately control the foodborne hazards that may be associated with the dairy products.

REFERENCE

Aboubaker, M. G., Enas, M. A., Salah, M. A., Said, K., Hesham, T. N., Ashraf, A. M., Fatim, T. G. Ilaria, B., & Ibrahim, M. E. (2016). Enterohemorrhagic *Escherichia coli* O157 in milk and dairy products from Libya: Isolation and molecular identification by partial sequencing of 16S rDNA. *Veterinary World*, *9*(11), 1184–1189.

Addo, K. K., Mensah, G. I., Aning, K. G., Nartey, N., Nipah, G. K., Bonsu, C., Akyeh, M. L., & Smits, H. L. (2011). Microbiological quality and antibiotic residues in informally marketed raw cow milk within the coastal savannah zone of Ghana. *Tropical Medicine and International Health*, *16*(2), 227–232. https://doi.org/10.1111/j.1365-3156.2010.02666.x

Adesiyun, A. A., Webb, L. A., & Romain, H. (1999). Phenotypic characteristics of Staphylococcus aureus strains isolated from milk and dairymen on dairy farms in Trinidad. *Israel Journal of Veterinary Medicine*, *54*(1), 1174–1181.

Ahmad, S., Gaucher, I., Rousseau, F., Beaucher, E., Piot, M., Grongnet, J. F., & Gaucheron, F. (2008). Effects of acidification on physico-chemical characteristics of buffalo milk: A comparison with cow's milk. *Food Chemistry*, *106*(1), 11–17. https://doi.org/10.1016/j.foodchem.2007.04.021

Ahmed, A. M., & Shimamoto, T. (2014). Isolation and molecular characterization of Salmonella enterica , *Escherichia coli* O157 : H7 and Shigella spp. from meat and dairy products in Egypt. *International Journal of Food Microbiology*, *168–169*, 57–

62. https://doi.org/10.1016/j.ijfoodmicro.2013.10.014

Aidoo, R., Nurah, G. K., Fialor, S. C., & Ohene-Yankyera, K. (2009). Determinants of Dairy Consumption Expenditure in Urban Communities Of Southern Ghana. *Journal of Science and Technology*, *29*(1).48-83

Akabanda, F., Owusu-Kwarteng, F., Glover, R. L. K., & K., T.-D. (2010). Microbiological Characteristics of Ghanaian Traditional Fermented Milk Product, Nunu. *Nature and Science Journal*, *8*(9), 178–187.

Aksu, H., Arun O.O., Aydin., A., & Ugur M. (1999). The presence of *Escherichia coli* O157: H7 in various foodstuffs of animal origin. *Pendik Vet Mikrobiyol Derg*, *30*, 77–81.

Alisarli M., & H.N, A. (2004). Investigation of *Escherichia coli* O157 in retail minced meat. *YYU Vet Fak Derg*, *15*, 65–69.

Altalhi, A. D., & Hassan, S. A. (2009). Bacterial quality of raw milk investigated by *Escherichia coli* and isolates analysis for specific virulence-gene markers. *Food Control*, *20*(10), 913–917. https://doi.org/10.1016/j.foodcont.2009.01.005

Álvarez-Ordóñez, A., Alvseike, O., Omer, M. K., Heir, E., Axelsson, L., Holck, A., & Prieto, M. (2013). Heterogeneity in resistance to food-related stresses and biofilm formation ability among verocytotoxigenic *Escherichia coli* strains. *International Journal of Food Microbiology*, *161*(3), 220–230. https://doi.org/10.1016/j.ijfoodmicro.2012.12.008

Amenu, K., Spengler, M., Markemann, A., & Zárate, A. V. (2014). Microbial quality of water in rural households of Ethiopia: Implications for milk safety and public health. *Journal of Health, Population and Nutrition*, *32*(2), 190–197. https://doi.org/10.3329/jhpn.v32i2.2614

American Academy of Pediatrics. (2014). Consumption of Raw or Unpasteurized Milk and Milk Products by Pregnant Women and Children. *Pediatrics*, *133*(1), 175–179. https://doi.org/10.1542/peds.2013-3502

Appiah, J. O. Y. (2012). Assessment of the risk of consuming milk / milk products contaminated with listeria monocytogenes from the informal markets. MPhil Thesis. University of Ghana. Accra, Ghana. https://hdl.handle.net/10568/21684

Arthur, A. B. (2016). Optimising the wagashie (a traditional cottage cheese) process and sensory quality. University of Ghana. Accra, Ghana MPil Thesis. http://197.255.68.203/handle/123456789/21881

Aryal, N., Halder, A., Tremblay, P. L., Chi, Q., & Zhang, T. (2016). Enhanced microbial electrosynthesis with three-dimensional graphene functionalized cathodes fabricated via solvothermal synthesis. *Electrochimica Acta*, *217*, 117–122. https://doi.org/10.1016/j.electacta.2016.09.063

Ateba, C. N., & Mbewe, M. (2011). Detection of *Escherichia coli* O157 : H7 virulence genes in isolates from beef , pork , water , human and animal species in the northwest province, South Africa : public health implications. *Research in*

Microbiology, *162*(3), 240–248. https://doi.org/10.1016/j.resmic.2010.11.008

Atiqah, N. (2017). Non-haemolytic enterotoxigenic *Bacillus cereus* strains from raw and pasteurized milk and milking utensils in Kelantan. *International Food Research Journal Malaysia*, *24*(1), 401–405.

Auldist, M. J., Johnston, K. A., White, N. J., Fitzsimons, W. P., & Boland, M. J. (2004). A comparison of the composition, coagulation characteristics and cheesemaking capacity of milk from Friesian and Jersey dairy cows. *Journal of Dairy Research*, *71*(1), 51–57. https://doi.org/10.1017/S0022029903006575

Aworh, O. C., & Muller, H. G. (1987). Cheese-making properties of vegetable rennet from sodom apple (Calotropis procera). *Food Chemistry*, *26*(1), 71–79. https://doi.org/10.1016/0308-8146(87)90168-3

Bae, Y. M., & Lee, S. Y. (2017). Effect of salt addition on acid resistance response of *Escherichia coli* O157:H7 against acetic acid. *Food Microbiology*, *65*, 74–82. https://doi.org/10.1016/j.fm.2016.12.021

Bagré, T. S., Sambe-Ba, B., Ibrahim, H. B., Tchamba, G. B., Dembélé, R., Wane, A. A., Bako, E., Traoré, A. S., Barro, N., & Gassama-Sow, A. (2017). Isolation and characterization of enteropathogenic and enterotoxinogenic *Escherichia coli* from dairy products consumed in Burkina Faso. *African Journal of Microbiology Research*, *11*(13), 537–545.

Baidoo, W., & Parry-Hanson Kunadu, A. (2018). Using low technology to improve the safety of informal-vended brukina – a fermented milk and millet smoothie in Ghana.

African Journal of Food, Agriculture, Nutrition and Development, 2(4) 13646–13663. https://doi.org/10.18697/ajfand.83.17620

Barlowska, J., Szwajkowska, M., Litwińczuk, Z., & Król, J. (2011). Nutritional Value and Technological Suitability of Milk from Various Animal Species Used for Dairy Production. *Comprehensive Reviews in Food Science and Food Safety*, *10*(6), 291–302. https://doi.org/10.1111/j.1541-4337.2011.00163.x

Bélanger, L., Garenaux, A., Harel, J., Boulianne, M., Nadeau, E., & Dozois, C. M. (2011). *Escherichia* colifrom animal reservoirs as a potential source of human extraintestinal pathogenic *E. coli*. *FEMS Immunology and Medical Microbiology*, *62*(1), 1–10. https://doi.org/10.1111/j.1574-695X.2011.00797.x

Belewu, M. A, Belewu, K. Y., & Nkwunonwo, C. C. (2005). Effect of biological and chemical preservatives on the shelf life of West African soft cheese. *African Journal of Biotechnology*, *4*(10), 1076–1079.

Boer, D. E., Heuvelink . E. (2000). Methods for the detection and isolation of Shiga toxin-producing *Escherichia coli*. *Journal of Applied Microbiology* 5(2), 133–148.

Bonyadian, M., Moshtaghi, H., & Mariam Akhavan Taheri. (2014). Molecular characterization and antibiotic resistance of enterotoxigenic and entero- aggregative *Escherichia coli* isolated from raw milk and unpasteurized cheeses. *Food Control* *5*(1), 29–34.

Brooks, J. C., Martinez, B., Stratton, J., Bianchini, A., Krokstrom, R., & Hutkins, R. (2012). Survey of raw milk cheeses for microbiological quality and prevalence of foodborne pathogens. *Food Microbiology, 31*(2), 154–158. https://doi.org/10.1016/j.fm.2012.03.013

Brooks, J. T., Sowers, E. G., Wells, J. G., Greene, K. D., Griffin, P. M., Hoekstra, R. M., & Strockbine, N. A. (2018). Non-O157 Shiga Toxin – Producing *Escherichia coli* Infections in the United States. *The Journal of Infectious Diseases*, 192(8), 1422–1429. https://doi.org/10.1086/466536

Brown, J. L., Ross, T., McMeekin, T. A., & Nichols, P. D. (1997). Acid habituation of *Escherichia coli* and the potential role of cyclopropane fatty acids in low pH tolerance. *International Journal of Food Microbiology, 37*(3), 163–173. https://doi.org/10.1016/S0168-1605(97)00068-8

Buchanan, R. L. (1996). Culturing enterohemorrhagic *Escherichia coli* in the presence and absence of glucose as a simple means of evaluating the acid tolerance culturing enterohemorrhagic. *Food Safety, 62*(11), 4009–4013.

Cabiscol, E., Tamarit, J., & Ros, J. (2000). Oxidative stress in bacteria and protein damage by reactive oxygen species. *International Microbiology, 3*(1), 3–8. https://doi.org/10.2436/im.v3i1.9235

Casey, P. G., & Condon, S. (2002). Sodium chloride decreases the bacteriocidal effect of acid pH on *Escherichia coli* O157:H45. *International Journal of Food Microbiology, 76*(3), 199–206. https://doi.org/10.1016/S0168-1605(02)00018-1

Castro, M. A. De. (2017). Dairy products consumption in Brazil is associated with socioeconomic and demographic factors : Results from the National Dietary Survey. *Revista Nutrition*, *30*(1), 79–90. https://doi.org/10.1590/1678-98652017000100008

Chaleshtori, F. S., Arani, N. M., Aghadavod, E., Naseri, A., & Chaleshtori, R. S. (2017). Molecular characterization of *Escherichia coli* recovered from traditional milk products in Kashan, Iran. *Veterinary World*, *10*(10), 1264–1268. https://doi.org/10.14202/vetworld.2017.1264-1268

Chen, J. (2015). Antimicrobial resistance of shiga toxin-producing *Escherichia coli*. In *Antimicrobial resistance and food safety: Methods and Techniques* (pp 19-36) Elsevier https://doi.org/10.1016/B978-0-12-801214-7.00002-8

Chen, Y., Wang, C., Hou, Wang, X. Shuo, Gali, B. & Chen, A. (2017). Effects of antibacterial compounds produced by Saccharomyces cerevisiae in Koumiss on pathogenic *Escherichia coli* and its cell surface characteristics. *Journal of Integrative Agriculture*, *16*(3), 742–748. https://doi.org/10.1016/S2095-3119(16)61516-2

Cheng, H. Y., Yu, R. C., & Chou, C. C. (2003). Increased acid tolerance of *Escherichia coli* O157:H7 as affected by acid adaptation time and conditions of acid challenge. *Food Research International*, *36*(1), 49–56. https://doi.org/10.1016/S0963-9969(02)00107-2

Claeys, W. L., Cardoen, S., Daube, G., De Block, J., Dewettinck, K., Dierick, K., De Zutter, L., Huyghebaert, A., Imberechts, H., Thiange, P., Vandenplas, Y., &

Herman, L. (2013). Raw or heated cow milk consumption: Review of risks and benefits. *Food Control*, *31*(1), 251–262. https://doi.org/10.1016/j.foodcont.2012.09.035

Coker, C. J., Crawford, R. A., Johnston, K. A., Singh, H., & Creamer, L. K. (2005). Towards the classification of cheese variety and maturity on the basis of statistical analysis of proteolysis data - A review. *International Dairy Journal*, *15*(6–9), 631–643. https://doi.org/10.1016/j.idairyj.2004.10.011

Cooke, E. M. (1974). *Escherichia coli* and *Klebsiella. Microbiology*, 32(8) pp216-226. http://www.cabdirect.org/abstracts/19742704379.html;jsessionid=0D3FCF364E983

Cortés-sánchez, A. D. J., & Salgado-cruz, M. D. P. (2017). *Escherichia coli* O157 : H7 in the context of foodborne diseases and public health. *Environmental and Experimental Biology*, 15, 191–200 DOI: 10.22364/eeb.15.19

Croatian standard institute. Microbiology of food and animal feeding stuff – Horizontal method for the enumeration of β – glucuronidase – positive Esherichia coli – Part 1: Colony-count technique at 44^{o}C using membranes and 5-bromo-4-chloro-3-indolyl-β-D-glucuronide *(ISO 16649-1:1st April 2001)*. Geneva Switzaland: International Organization of Standardization

Croxen, M. A., & Finlay, B. B. (2010). Molecular mechanisms of *Escherichia coli* pathogenicity. *Nature Reviews Microbiology*, *8*(1), 26–38. https://doi.org/10.1038/nrmicro2265

Dadie, A., Tagro, G., Ocho Anin, L., Dako, E., Dje, M., & Dosso, M. (2010). Gastroenteritis *E. coli* carried by milk products sold in the street of Abidjan, Côte d'Ivoire. *European Journal of Scientific Research*, *39*(1)15-67.

De Melo, C. B., De Sá, M. E. P., Dos Reis Souza, A., De Oliveira, A. M., Moacyr, P., Mota, P. C., Campani, P. R., Luna, J. O., Pinto, S. C., Schwingel, F. F., McManus, C., & Seixas, L. (2014). Bacteria in dairy products in baggage of incoming travelers, Brazil. *Emerging Infectious Diseases*, *20*(11), 1933–1935. https://doi.org/10.3201/eid2011.131422

Desmarchelier, P. (2011). *Escherichia coli*. *International Dairy Journal*, 4(5)60–66.

Dilielo, L. R. (1982). Methods in food and dairy microbiology. *Trends in Biotechnology*. 1(2) 65-66

Donkor, E., Aning, K., & Quaye, J. (2007). Bacterial contaminations of informally marketed raw milk in Ghana. *Ghana Medical Journal*, *41*(2), 58–61. https://doi.org/10.4314/gmj.v41i2.55302

Dossou, J., Hounzangbe-Adote, S., & Soule, H. (2006). Production et transformation du lait frais en fromage peulh au benin. *Guide De Bonnes Pratiques* 5(8)90-209

Elhadidy, M., & Álvarez-Ordóñez, A. (2016). Diversity of survival patterns among *Escherichia coli* O157: H7 genotypes subjected to food-related stress conditions. *Frontiers in Microbiology*, *7*(3), 1–10. https://doi.org/10.3389/fmicb.2016.00322

Ellingson, J. A. Y. L. E., Anderson, J. L., Koziczkowski, J. J., Radcliff, R. O. Y. P., Sloan, S. J., Allen, S. E., & Sullivan, N. M. (2005). Detection of viable mycobacterium avium subsp. paratuberculosis in retail pasteurized whole milk by two culture methods and PCR. *Journal of Food Protection*, *68*(5), 966–972.

Fang, F. (2013). Antibiotic and ROS linkage questioned. *Nature Biotechnology*. 31:415–416. http://dx.doi.org/10.1038/nbt.2574

FAO. (2012). A Living from livestock. *A guide to sustainable production intensification*. (available at http://faostat. fao.org/, docrep/015/i2744e/i2744e00.pdf accessed, 11th August 2012)

FAOSTAT. (2010) Characterization and value addition to local breeds and their products in Africa. Available from: http://faostat.fao.org/. Accessed 24th April, 2011. *Vailable from: Http://Faostat.Fao.Org/., Browse/Q/QL/E*.

FAOSTAT. (2018). Animal Satistics. *Animal Science*. Retrieved from https://faostat3. *Fao.Org/ Browse/Q/QL/E*.3(4)55-68

Farzan, S. F., Karagas, M. R., & Chen, Y. (2013). In utero and early life arsenic exposure in relation to long-term health and disease. *Toxicology and Applied Pharmacology*, *272*(2), 384–390. https://doi.org/10.1016/j.taap.2013.06.030

Ferro, M. M., Tedeschi, L. O., & Atzori, A. S. (2017). The comparison of the lactation and milk yield and composition of selected breeds of sheep and goats. *Translational Animal Science*, *1*(4), 498–506. https://doi.org/10.2527/tas2017.0056

Fink, A. L. (2018). Chaperone-Mediated Protein Folding. *Journals Physiology organization 79*(2), 425–449.

Foster, J. (2001). Acid stress responses of *Salmonella* and *E. coli:* Survival mechanisms, regulation, and implications for pathogenesis. *Journal of Microbiology*, *39*, 89–94.

Freedman, B. (1977). Milk quality in sanitarials handbook: *Theory and administrative practice for environmental health* (4th ed.). Peerless Publishing, New Oreleans, USA, pp: 564-589.

Gemechu, T., Beyene, F., & Eshetu, M. (2014). Handling practices and microbial quality of raw cow ' s milk produced and marketed in Shashemene town , southern Ethiopia. *International Journal of Agric Soil Science*, 2 (9):153–162. https://doi.org/10.5897/ISABB-JFAS2014.0017

Gidiglo, K. F. (2014). Milk production and marketing in Ghana: The case of Accra plains. *Journal of Biology, Agriculture and Healthcare*, *4*(16), 60–64.

Gomes, T. A. T., Elias, W. P., Scaletsky, I. C. A., Guth, B. E. C., Rodrigues, J. F., Piazza, R. M. F., Ferreira, L. C. S., & Martinez, M. B. (2016). Diarrheagenic *Escherichia coli*. *Brazilian Journal of Microbiology*, *47*, 3–30. https://doi.org/10.1016/j.bjm.2016.10.015

Gran, H. M., Wetlesen, A., Mutukumira, A. N., Rukure, G., & Narvhus, J. A. (2003). Occurrence of pathogenic bacteria in raw milk, cultured pasteurised milk and naturally soured milk produced at small-scale dairies in Zimbabwe. *Food Control*, *14*(8), 539–544. https://doi.org/10.1016/S0956-7135(02)00117-2

Guion, C. E., Ochoa, T. J., Walker, C. M., Barletta, F., & Cleary, T. G. (2008). Detection of diarrheagenic *Escherichia coli* by use of melting-curve analysis and real-time multiplex PCR. *Journal of Clinical Microbiology*, *46*(5), 1752–1757. https://doi.org/10.1128/JCM.02341-07

Haiqing, S., Smriti. S., Baker K. N. K., Minnich. S. A. J., Hovde. C. E., & Besser. T. (2016). Standardized *Escherichia coli* O157:H7 exposure studies in cattle provide evidence that bovine factors do not drive increased summertime colonization. *American Society forMicrobiology*, *82*(3), 964–971.

Hasman, H., Schembri, M. A., & Klemm, P. (2000). Antigen 43 and type 1 fimbriae determine colony morphology of *Escherichia coli* K-12. *Journal of Bacteriology*, *182*(4), 1089–1095. https://doi.org/10.1128/JB.182.4.1089-1095.2000.Updated

Hayes, M. C., Ralyea, R. D., Murphy, S. C., Carey, N. R., Scarlett, J. M., & Boor, K. J. (2001). Identification and characterization of elevated microbial counts in bulk tank raw milk. *Journal of Dairy Science*, *84*(1), 292–298.

Heiman, K. E., Mody, R. K., Johnson, S. D., Griffin, P. M., & Hannah Gould, L. (2015). *Escherichia coli* O157 Outbreaks in the United States. *Emerging Infectious Diseases*, *21*(8), 1293–1301. https://doi.org/10.3201/eid2108.141364

Hendriksen, R. S. (2003). Isolation and identification of enterohaemorrhagic *Escherichia coli* O157. *Global Salm-Surv, A Global Salmonella Surveillance and Laboratory Support Project of the World Health Organization*, *1*(2), 236–237.

Herrmann, K., & Carroll, K. (2014). An exclusively human milk diet reduces necrotizing enterocolitis. *Breastfeeding Medicine*, *9*(4), 184–190. https://doi.org/10.1089/bfm.2013.0121

Hess, J. M., Jonnalagadda, S. S., & Slavin, J. L. (2016). Dairy foods: Current evidence of their effects on bone, cardiometabolic, cognitive, and digestive health. *Comprehensive Reviews in Food Science and Food Safety*, *15*(2), 251–268. https://doi.org/10.1111/1541-4337.12183

Hessain, A. M., Al-Arfaj, A. A., Zakri, A. M., El-Jakee, J. K., Al-Zogibi, O. G., Hemeg, H. A., & Ibrahim, I. M. (2015). Molecular characterization of *Escherichia coli* O157: H7 recovered from meat and meat products relevant to human health in Riyadh, Saudi Arabia. *Saudi Journal of Biological Sciences*, *22*(6), 725–729. https://doi.org/10.1016/j.sjbs.2015.06.009

Hoddinott, J., Headey, D., & Dereje, M. (2015). Cows, missing milk markets, and nutrition in rural Ethiopia. *Journal of Development Studies*, *51*(8), 958–975. https://doi.org/10.1080/00220388.2015.1018903

Hsin-Yi, C., & Chou, C. C. (2001). Acid adaptation and temperature effect on the survival of *E. coli* O157:H7 in acidic fruit juice and lactic fermented milk product. *International Journal of Food Microbiology*, *70*(1–2), 189–195. https://doi.org/10.1016/S0168-1605(01)00538-4

Https://www.britannica.com/science/E-coli. (2016). *E. coli*. *Encyclopæd*. Accessed 1st March 2018. phil.cdc.gov/Details.aspx?pid=10068

Huang, H. W., Lung, H. M., Yang, B. B., & Wang, C. Y. (2014). Responses of microorganisms to high hydrostatic pressure processing. *Food Control*, *40*(1), 250–259. https://doi.org/10.1016/j.foodcont.2013.12.007

Ivbade, A., Ojo, O. E., & Dipeolu, M. A. (2014). Shiga toxin-producing *Escherichia coli* O157:H7 in milk and milk products in Ogun State, Nigeria. *Veterinaria Italiana*, *50*(3), 185–191. https://doi.org/10.12834/VetIt.129.2187.1

Jerry, A.-A., Hafusatu, M., Emeruwa, H. O., Kant, O. F., & Bright, Q. (2020). Quality changes during storage of Burkina (a Millet and Milk-based) drink. *Asian Food Science Journal*, *15*(2), 1–5. https://doi.org/10.9734/afsj/2020/v15i230146

Johnson, J. R., Murray, A. C., Kuskowski, M. A., Schubert, S., Prère, M. F., Picard, B., Colodner, R., Raz, R., Bodison, S., Cockerill, F. R., Da Cunha, C. A., Echeverria, P., Simasathien, S., Gaastra, W., Hall, L. M. C., Klein, M., Lockman, H. A., Omer, I., Schwebke, J. R., … Winokur, P. L. (2005). Distribution and characteristics of *Escherichia coli* clonal group A. *Emerging Infectious Diseases*, *11*(1), 141–145. https://doi.org/10.3201/eid1101.040418

Kai, T. M. K., & Aotearoa, A. (2009). Microbiological risk assessment of raw cow milk. *Food Standards Fsanz*. 2(3)40-69

Kammerlehner, J. (2009). Cheese technology. *Food Policy*, *57*(2), 930.

Karamba, W. R., Quiñones, E. J., & Winters, P. (2011). Migration and food consumption patterns in Ghana. *Food Policy*, *36*(1), 41–53. https://doi.org/10.1016/j.foodpol.2010.11.003

Kaur, P., & Asea, A. (2017). Loss of biofilm formation in an emerging foodborne pathogen enteroaggregative *Escherichia coli* (EAEC) under acid stress. *Cell Science 8*(1), 1–7. https://doi.org/10.4172/2157-7013.

Kim, H. J., Yong, H. I., Park, S., Kim, K., Choe, W., & Jo, C. (2015). Microbial safety and quality attributes of milk following treatment with atmospheric pressure encapsulated dielectric barrier discharge plasma. *Food Control*, *47*, 451–456. https://doi.org/10.1016/j.foodcont.2014.07.053

Kim, K. M., Choi, H. S., Choi, M.-J., & Chung, H. Y. (2015). Calcium and vitamin D supplementations: 2015 Position statement of the korean society for bone and mineral research. *Journal of Bone Metabolism*, *22*(4), 143. https://doi.org/10.11005/jbm.2015.22.4.143

Kolbus, A., Blázquez-Domingo, M., Carotta, S., Bakker, W., Luedemann, S., Von Lindern, M., Steinlein, P., & Beug, H. (2003). Cooperative signaling between cytokine receptors and the glucocorticoid receptor in the expansion of erythroid progenitors: Molecular analysis by expression profiling. *Blood*, *102*(9), 3136–3146. https://doi.org/10.1182/blood-2003-03-0923

Kumar, K. S., & Kannan, P. (2018). Prevalence of *Escherichia coli* and *Salmonella spp* . in Captive Niligiri Langur (Trachypithecus johnii) in south India. *International Journal of Current Microbiology and Applied Sciences,* 7(6)366-706 https://doi.org/10.20546/ijcmas.

Kunadu, A. P., Aboagye, E. F., Colecraft, E. S. I. K., Otoo, G. E., Adjei, M. Y. B., Acquaah, E., Afrifa-Anane, E., & Amissah, J. G. N. (2019). Low consumption of indigenous fresh dairy products in Ghana attributed to poor hygienic quality. *Journal of food Protection, 82*(2), 276–286. https://doi.org/10.4315/0362-028X.JFP-18-146

Lee, S. Y., & Kang, D. H. (2016). Survival mechanism of *Escherichia coli* O157: H7 against combined treatment with acetic acid and sodium chloride. *Food Microbiology*, *55*, 95–104. https://doi.org/10.1016/j.fm.2015.10.021

Liaqat, I., & Sabri, A. N. (2010). Biofilm, dental unit water line and its control. *In African Journal Of Clinical And Experimental Microbiology,* 12 (1), 55-109. http://www.ajol.info/journals/ajcem

Liu, Yuejiao, Singh, P., & Mustapha, A. (2018). High-resolution melt curve PCR assay for specific detection of *E. coli* O157:H7 in beef. *Food Control*, *86*, 275–282. https://doi.org/10.1016/j.foodcont.2017.11.025

Liu, Yuping, Tang, H., Lin, Z., & Xu, P. (2015). Mechanisms of acid tolerance in bacteria and prospects in biotechnology and bioremediation. *Biotechnology Advances*, *33*(7), 1484–1492. https://doi.org/10.1016/j.biotechadv.2015.06.001

Lopez-saucedo, C., Cerna, J., & Villegas-sepulveda, N. (2003). Polymerase chain reaction to detect diverse loci associated with *Escherichia coli*. *Emerging Infectious Diseases*, *9*, 127–131.

Lucht, J. M., & Bremer, E. (1994). Adaptation of *Escherichia coli* to high osmolarity environments: osmoregulation of the high-affinity glycine betaine transport system proU. *FEMS Microbiol*ogy, *14(1)*, 3–20.

Luciani, M., Di Febo, T., Zilli, K., Di Giannatale, E., Armillotta, G., Manna, L., Minelli, F., Tittarelli, M., & Caprioli, A. (2016). Rapid detection and isolation of *Escherichia coli* O104:H4 from milk using monoclonal antibody-coated magnetic beads. *Frontiers in Microbiology*, *7*(2), 1–7. https://doi.org/10.3389/fmicb.2016.00942

Lues, J. F. R., Venter, P., & Van Der Westhuizen, H. (2003). Enumeration of potential microbiological hazards in milk from a marginal urban settlement in central South Africa. *Food Microbiology*, *20*(3), 321–326. https://doi.org/10.1016/S0740-0020(02)00128-4

Luna-Gierke, R. E., Griffin, P. M., Gould, L. H., Herman, K., Bopp, C. A., Strockbine, N., & Mody, R. K. (2014). Outbreaks of non-O157 Shiga toxin-producing *Escherichia coli* infection: USA. *Epidemiology and Infection*, *142*(11), 2270–2280. https://doi.org/10.1017/S0950268813003233

Mahmuda, A., Mohammed, A. A., Alayande, M. O., Habila, Y. I., Lawal, M. D., Usman, M., & Raji, A. A. (2014). Prevalence and distribution of gastrointestinal parasites of working camels in Sokoto metropolis. *Veterinary World, EISSN. 7*, 108–112. https://doi.org/10.14202/vetworld.2014.108-112

Martin, M. L., Shipman, L. D., Potter, M. E., Wachsmuch, I. K., Wells, J. G., Hedberg, K., Tauxe, R. V., Davis, P., Arnoldi, J., & Tilleli, J. (1986). Isolation of *Escherichia*

coli O157:H7 from dairy cattle associated with two cases of hemolytic uremic syndrome. *The Lancet Regional Health, 2,* 1043-1078.

Mason, M. G., Shepherd, M., Nicholls, P., Dobbin, P. S., Dodsworth, K. S., Poole, R. K., & Cooper, C. E. (2009). Cytochrome bd confers nitric oxide resistance to *Escherichia coli*. *Nature Chemical Biology,* 5 (9)94–96. https://doi.org/10.1038/nchembio.135

Ministry of Food and Agriculture. (2010). Medium Term Agriculture Sector Investment Plan (METASIP). Ministry of Food and Agriculture, Ghana. Retrieved January 20, 2017, from http://mofa.gov.gh/site/?page_id=2754

Mullins, G., Rey, B., Nokoe, S., & Shapiro, B. (1994). A research methodology for characterizing dairy product consumption systems, market-oriented smallholder dairying research. *American Journal of Food Technology,* 5(2)56-99.

Muraro, A., Høst, A., Meyer, R., Kuslys, M., Muraro, A., & Høst, A. (2017). Controversies on special products for managing cow's milk protein allergy in infants : Safety and Suitability. *European Medical Journal,* 7, (1)46–51.

Mwini, G. A., & Darkwa, S. (2016). Microbial contamination associated with "wagashi-cheese" production in Sissala east district- Ghana. *Journal of Food Research, 5*(4), 11. https://doi.org/10.5539/jfr.v5n4p11

Nanu, E., Latha, C., Sunil, B., M, T., Prejit, ., & Venon, K. V. (2007). Quality assurance and public health safety of raw milk at the production point. *American Journal of*

Food Technology, *2*, 145–152.

Ngigi, M. (2003). The case of smallholder dairying in eastern Africa. *Successes in African Agriculture:*.5(6), 23-40

Nguyen, Y., & Sperandio, V. (2012). Enterohemorrhagic *E. coli* (EHEC) pathogenesis. *Frontiers in cellular and infection microbiology*, 2, 90-109. https://doi.org/10.3389/fcimb.2012.00090

O'Bryan, C. A., Pendleton, S. J., Ricke, S. C., & Crandall, P. G. (2017). Interventions to reduce shiga toxin-producing *Escherichia coli* on beef carcasses at slaughter. *Food and feed safety systems and analysis*, 1,(5) 195-212 https://doi.org/10.1016/B978-0-12-811835-1.00011-7

Obi, C., E, G., Bessong, P., Villiers, B. De, Hoosen, A., Igumbor, E., & Potgieter, N. (2004). Gene encoding virulence markers among *Escherichia coli* isolates from diarrhoeic stool samples and river sources in rural Venda communities of South Africa. *African Journal of Microbiology Research*, *30*(1), 88–96.

Obodai, M., & Dodd, C. (2006). Characterization of dominant microbiota of a Ghanaian fermented milk product, nyarmie, by culture-and nonculture-based methods. *Applied Microbiology*, *100*, 1355–1363.

Okantah, S., Obese, F., Oddoye, O., Karikari, P., Gyawu, P., & Bryant, M. (2006). The effect of farm (herd) and season of calving on the reproductive performance of Sanga cows in smallholder peri-urban dairy farms in the Accra plains. *Ghana Journal of Agricultural Science*, *38*(2) 67-111.

https://doi.org/10.4314/gjas.v38i2.2098

Olalekan, A., Agboolaa, T., Downsa Gordon O.B. (2019). Macroinvertebrates as indicators of ecological conditions in the rivers of KwaZulu-Natal, South Africa. *Ecological Indicators, 106*(2) 1054-1065.

Olalokun, E.A. (1996). Milk production in West Africa: objectives and research nisin for the preservation of nono, a Nigerian fermented milk product. *Journal of Food Processing and Preservation, 20,* 71–78.

Oliver, S. P., Boor, K. J., Murphy, S. C., & Murinda, S. E. (2009). Food safety hazards associated with consumption of raw milk. *Foodborne pathogens and disease*, *6*(7), 793–806. https://doi.org/10.1089/fpd.2009.0302

Olsvik, Ø., Wasteson, Y., Lund, A., & Hornes, E. (1991). Pathogenic *Escherichia coli* found in food. *International Journal of Food Microbiology*, *12*(1), 103–113. https://doi.org/10.1016/0168-1605(91)90051-P

Ombarak, R. A., Hinenoya, A., Awasthi, S. P., Iguchi, A., Shima, A., Elbagory, A. R. M., & Yamasaki, S. (2016). Prevalence and pathogenic potential of *Escherichia coli* isolates from raw milk and raw milk cheese in Egypt. *International Journal of Food Microbiology*, *221*, 69–76. https://doi.org/10.1016/j.ijfoodmicro.2016.01.009

Omer, M. K., Alvseike, O., Holck, A., Axelsson, L., Prieto, M., Skjerve, E., & Heir, E. (2010). Application of high pressure processing to reduce verotoxigenic *E . coli* in two types of dry-fermented sausage. *Meat Science, 86*(4), 1005–1009. https://doi.org/10.1016/j.meatsci.2010.08.008

Omore, A., Staal, S. J., Osafo, E. L. K., Kurwijilla, L., Barton, D., Mdoe, N., Nurah, G., & Aning, G. (2004). Market mechanisms, efficiency, processing and public health risks in peri-urban dairy product markets: Synthesis of findings for Ghana and Tanzania. *International Livestock Institute*, 3(4) 39-108

Omore, A., Staal, S. J., Wanyoike, F., Osafo, E. L. K., Kurwijila, L., Barton, D., Mdoe, N., Nura, G., & Aning, G. (2009). Market mechanisms and efficiency in urban dairy products markets in Ghana and Tanzania. *International Livestock Institute*, 6(7)1–57. papers3://publication/uuid/551D499C-C842-47A2-8680-2EA086E000B1

Owusu-Kwarteng. James 1, Wuni. Alhassan, F. A. 1 and L. J. 3 I. (2018). Prevalence and Characteristics of *Listeria monocytogenes* Isolates in Raw Milk , Heated Milk. *Beverages*, *13*, 1–10. https://doi.org/10.3390/beverages4020040

Owusu-Kwarteng, J., Wuni, A., Akabanda, F., Tano-Debrah, K., & Jespersen, L. (2017). Prevalence, virulence factor genes and antibiotic resistance of *Bacillus cereus* sensu lato isolated from dairy farms and traditional dairy products. *BMC Microbiology*, *17*(1), 9–16. https://doi.org/10.1186/s12866-017-0975-9

Pal, M. (2017). *Escherichia coli* 0517 : H7 : An emerging bacterial zoonotic food borne pathogen of global significance *Escherichia coli 0517 : H7 : An emerging bacterial zoonotic food*. *January 2016*. 2(5) 63-111

Paneto, B. R., Schocken-Iturrino, R. P., Macedo, C., Santo, E., & Marin, J. M. (2007). Occurrence of toxigenic *Escherichia coli* in raw milk cheese in Brazil. *Arquivo Brasileiro de Medicina Veterinaria e Zootecnia*, *59*(2), 508–512.

https://doi.org/10.1590/S0102-09352007000200035

Pappa, E. C., Bontinis, T. G., Tasioula-Margari, M., & Samelis, J. (2017). Microbial quality of and biochemical changes in fresh soft, acid-curd Xinotyri cheese made from raw or pasteurized goat's milk. *Food Technology and Biotechnology*, *55*(4), 496–510. https://doi.org/10.17113/ftb.55.04.17.5338

Parry-Hanson, A. A., Jooste, P. J., & Buys, E. M. (2010). Relative gene expression in acid-adapted *Escherichia coli* O157:H7 during lactoperoxidase and lactic acid challenge in Tryptone Soy Broth. *Microbiological Research*, *165*(7), 546–556. https://doi.org/10.1016/j.micres.2009.11.003

Parry-Hanson, A., Jooste, P., & Buys, E. (2009a). Inhibition of *Escherichia coli* O157:H7 in commercial and traditional fermented goat milk by activated lactoperoxidase. *Dairy Science and Technology*, *89*(6), 613–625. https://doi.org/10.1051/dst/2009042

Parry-Hanson, A., Jooste, P., & Buys, E. (2009b). Inhibition of *Escherichia coli* O157:H7 in commercial and traditional fermented goat milk by activated lactoperoxidase. *Dairy Science and Technology*, *89*(6), 613–625. https://doi.org/10.1051/dst/2009042

Parry-Hanson, A., Jooste, P. J., & Buys, E. M. (2009c). The influence of lactoperoxidase, heat and low pH on survival of acid-adapted and non-adapted *Escherichia coli* O157:H7 in goat milk. *International Dairy Journal*, *19*(6–7), 417–421. https://doi.org/10.1016/j.idairyj.2009.02.005

Parry-Hanson, K. A., Aboagye, E. F., Colecraft, E. S. I. K., Otoo, G. E., Adjei, M. Y. B., Acquaah, E., Afrifa-anane, E., & Amissah, J. G. N. (2019). Low consumption of

indigenous fresh dairy products in Ghana attributed to poor hygienic quality. *Journal of Food Production, 82*(2), 276–286. https://doi.org/10.4315/0362-028X.JFP-18-146

Parry-Hanson Kunadu, A., Holmes, M., Miller, E. L., & Grant, A. J. (2018). Microbiological quality and antimicrobial resistance characterization of *Salmonella spp*. in fresh milk value chains in Ghana. *International Journal of Food Microbiology, 277*, 41–49. https://doi.org/10.1016/j.ijfoodmicro.2018.04.025

Pawłowska, B., & Sobieszczańska, B. M. (2017). Intestinal epithelial barrier: The target for pathogenic *Escherichia coli*. *Advances in Clinical and Experimental Medicine, 26*(9), 1437–1445. https://doi.org/10.17219/acem/64883

Reale, A., Di Renzo, T., Rossi, F., Zotta, T., Iacumin, L., Preziuso, M., Parente, E., Sorrentino, E., & Coppola, R. (2015). Tolerance of *Lactobacillus casei, Lactobacillus paracasei* and *Lactobacillus rhamnosus* strains to stress factors encountered in food processing and in the gastro-intestinal tract. *LWT - Food Science and Technology, 60*(2), 721–728. https://doi.org/10.1016/j.lwt.2014.10.022

Renuka, N., Sathian, C. T., Sujatha, S., & Deepa, S. (2009). Impact of family income on consumption of livestock products at kalpetta, kerala. *Veterinary World, 2*(8), 323–324.

Reshes, G., Vanounou, S., Fishov, I., & Feingold, M. (2008). Cell shape dynamics in *Escherichia coli*. *Biophysical Journal, 94*(1), 251–264. https://doi.org/10.1529/biophysj.107.104398

Rovira, R. F. (2015). La leche y los productos lácteos: Fuentes dietéticas de calcio. *Nutricion Hospitalaria*, *31*, 1–9. https://doi.org/10.3305/nh.2015.31.sup2.8676

Ryu, J. H., & Beuchat, L. R. (1998). Influence of acid tolerance responses on survival, growth, and thermal cross-protection of *Escherichia coli* O157:H7 in acidified media and fruit juices. *International Journal of Food Microbiology*, *45*(3), 185–193. https://doi.org/10.1016/S0168-1605(98)00165-2

Sainz, T., Pérez, J., Villaseca, J., Hernández, U., Eslava, C., Mendoza, G., & Wacher, C. (2005). Survival to different acid challenges and outer membrane protein profiles of pathogenic *Escherichia coli* strains isolated from pozol, a Mexican typical maize fermented food. *International Journal of Food Microbiology*, *105*(3), 357–367. https://doi.org/10.1016/j.ijfoodmicro.2005.04.017

Sampane-Donkor, E. (2002). A study of milk quality and public health hazards in the smallholder peri-urban dairy marketing system in Ghana. MPhil Thesis University of Ghana. pdfs.semanticscholar.org

Sancak, Y. C., Sancak, H., & Isleyici, O. (2015). Presence of *Escherichia coli* O157 and O157:H7 in raw milk and Van herby cheese. *Bulletin of the Veterinary Institute in Pulawy*, *59*(4), 511–514. https://doi.org/10.1515/bvip-2015-0076

Sanz, L., Ramos, E., De, G., Adarve, T., & Dı, J. (2009). Analysis Composition of goat and cow milk produced under similar conditions and analyzed by identical methodology. *Journal of Food Composition, 22*, 322–329. https://doi.org/10.1016/j.jfca.2008.10.020

Saxena, T., Kaushik, P., & Krishna Mohan, M. (2015). Prevalence of *E. coli* O157: H7 in water sources: An overview on associated diseases, outbreaks and detection methods. *Diagnostic Microbiology and Infectious Disease*, *82*(3), 249–264. https://doi.org/10.1016/j.diagmicrobio.2015.03.015

Schlesser, J. E., Gerdes, R., Ravishankar, S., Madsen, K., Mowbray, J., & Teo, A. Y. (2006). Survival of a five-strain cocktail of *Escherichia coli* O157:H7 during the 60-day aging period of cheddar cheese made from unpasteurized milk. *Journal of Food Protection Protion*, *60*(5), 990–998.

Sessou, P., Farougou, S., Azokpota, P., Youssao, I., Yèhouénou, B., & Ahounou, S. Sohounhloue, C. (2013). Inventaire et analyse des pratiques endogènes de conservation du wagashi, un fromage traditionnel produit au Bénin. *International Journal of Biological and Chemical Science*, *7*, 938–952.

Shaker, E. M., & Elaref, M. Y. (2015). Detection of raw buffalo's milk adulteration in sohag governorate. *Assiut Veterinary Medicals*, *61*(144), 38–45.

Singh, P., & Gandhi, N. (2015). Milk preservatives and adulterants: processing, regulatory and safety issues. *Food reviews international*, *31*(3), 236–261. https://doi.org/10.1080/87559129.2014.994818

Sintayehu, Y., Fekadu, B., Azage, T., & Berhanu, G. (2008). Dairy production, processing and marketing systems of Shashemene-Dilla area, South Ethiopia. IPMS (Improving Productivity and Market Success) of Ethiopia farmers project working: *International Livestock Research,* 62(1), 1-55

Slonczewski, J. L., Fujisawa, M., Dopson, M., & Krulwich, T. A. (2009). Cytoplasmic pH measurement and homeostasis in bacteria and archaea. *Advances in Microbial Physiology*, *55*, 1–79.

Soomro, A. H., Arain, M. A., Khaskheli., M., & Bhutto., B. (2002). Isolation of *Escherichia coli* from raw milk and milk products in relation to public health sold under market conditions at tandojam, pakistan. *Pakistan journal of nutrition*, *1*(3), 151–152. https://doi.org/10.3923/pjn.2002.151.152

Sudda, M. M., Mtenga, A. B., Kusiluka, L. J., & Kassim, N. (2016). Prevalence and antibiotic susceptibility of *Escherichia coli* and *Salmonella spp*. isolated from milk of zero grazed cows in Arusha City. *African Journal of Microbiology Research*, *10*(46), 1944–1951. https://doi.org/10.5897/AJMR2016.8209

Syrstad, O. (2018). Milk yield and lactation length in tropical cattle. *World Animal Review*, 74/75: 68–72

Tankoano, A., Kaboré, D., Savadogo, A., Soma, A., Fanou-Fogny, N., Compaore-Sereme, D., Hounhouigan, J. D., & Sawadogo-Lingani, H. (2016). Evaluation of microbiological quality of raw milk, sour milk and artisanal yoghurt from Ouagadougou, Burkina Faso. *African Journal of Microbiology Research, 10*(16), 535–541. https://doi.org/10.5897/AJMR2015.7949

Tanzin, T., Nazir, K. H. M. N. H., Zahan, M. N., Parvej, S., & Zesmin, K. (2016). Antibiotic resistance profile of bacteria isolated from raw milk samples of cattle and buffaloes. *Journal of Advanced Veterinary and Animal Research*, 3,(1)62–67.

https://doi.org/10.5455/javar.2016.c133

Tokuşoğlu, Ö., Aycan, S., Akalin, S., Koçak, S., & Ersoy, N. (2004). Simultaneous differential pulse polarographic determination of cadmium, lead, and copper in milk and dairy products. *Journal of Agricultural and Food Chemistry*, *52*(7), 1795–1799.

Tripaldi, C., Palocci, G., Miarelli, M., Catta, M., Orlandini, S., Amatiste, S., Bernardini, R. Di, & Catillo, G. (2010). Effects of mastitis on buffalo milk quality. *Journal of Animal Science*, *23*(10), 1319–1324.

Valverde, C. N., & Quesada Gómez, J. M. (2015). Vitamina D, determinante de la salud ósea y extra ósea; Importancia de su suplementación en la leche y derivados. *Nutricion Hospitalaria*, *31*, 18–25. https://doi.org/10.3305/nh.2015.31.sup2.8678

Van Giau, V., Nguyen, T. T., Nguyen, T. K. O., Le, T. T. H., & Nguyen, T. D. (2016). A novel multiplex PCR method for the detection of virulence-associated genes of *Escherichia coli* O157:H7 in food. *Biotechnology*, *6*(1), 1–8. https://doi.org/10.1007/s13205-015-0319-0

Velliou, E. G., Van Derlinden, E., Cappuyns, A. M., Nikolaidou, E., Geeraerd, A. H., Devlieghere, F., & Van Impe, J. F. (2011). Towards the quantification of the effect of acid treatment on the heat tolerance of *Escherichia coli* K12 at lethal temperatures. *Food Microbiology*, *28*(4), 702–711. https://doi.org/10.1016/j.fm.2010.06.007

Velliou, Eirini G., Van Derlinden, E., Cappuyns, A. M., Goossens, J., Geeraerd, A. H., Devlieghere, F., & Van Impe, J. F. (2011). Heat adaptation of *Escherichia coli* K12:

Effect of acid and glucose. *Procedia Food Science, 1*, 987–993. https://doi.org/10.1016/j.profoo.2011.09.148

Walton, G. M. and W. F. C. (1998). Understanding economics today. *Review of World Economics*, 144, 32-54.

Wang, G., Clark, C. G., & Rodgers, F. G. (2002). Detection in *Escherichia coli* of the genes encoding the major virulence factors, the genes defining the O157 : H7 serotype, and components of the type 2 shiga toxin family by multiplex PCR. *Journal of Clinical Microbiology 40*(10), 3613–3619. https://doi.org/10.1128/JCM.40.10.3613

Wu, W., Zhao, S., Mao, Y., Fang, Z., Lu, X., & Zeng, L. (2015). A sensitive lateral flow biosensor for *Escherichia coli* O157: H7 detection based on aptamer mediated strand displacement amplification. *Analytica Chimica Acta*, *861*, 62–68. https://doi.org/10.1016/j.aca.2014.12.041

Wullschleger, S. M. (2009). Biodiversity and microbial safety of artisanal malian sour milk fènè and development of adapted starter cultures for controlled production. *International dairy journal*, 29:28 –35. https:// doi.org/10.1016/j.idairyj.2012.08.001

Yahuza, M. L. (2001). Small - holder dairy production and marketing constrains in Nigeria. In: Rangnekegr D, Thorpe W (Eds). Proceedings of a south – south workshop held at national dairy development board (NDDB). Anand, India, 13 – 16 March 2001, and ILRI (International livestock research institute), Nairobi, Kenya.

Yoon, Y., Lee, S., & Choi, K. H. (2016). Microbial benefits and risks of raw milk cheese. *Food Control*, *63*, 201–215. https://doi.org/10.1016/j.foodcont.2015.11.013

Zaheer R, Dugat-Bony E, Holman D, Cousteix E, X. Y. (2017). Changes in bacterial community composition of *Escherichia coli* O157:H7 super-shedder cattle occur in the lower intestine. *PLOS ONE*, *2*(1), 87-156

Zecchini, M., & Cantàfora, A. F. A. (2010). Produrre latte in Africa subsahariana con particolare riferimento ai paesi dell'Africa centro-occidentale. *SIVtro - VSF Italia*, cited 12th Oct. 2016. http://www.dimevet.unimi.it/ecm/home/rapporti-internazionali/attivita-di-cooperazione-allo-sviluppo.

Zhang, Y., Yan, C., Yang, H., Yu, J., & Wei, H. (2017). Rapid and selective detection of *E. coli* O157:H7 combining phagomagnetic separation with enzymatic colorimetry. *Food Chemistry*, *234*, 332–338. https://doi.org/10.1016/j.foodchem.2017.05.013

Zinnah, M. A., Bari, M. R., Islam, M. T., Hossain, M. T., Rahman, M. T., Haque, M. H., Babu, S. A. M., Ruma, R. P., & Islam, M. A. (2007). Characterization of *Escherichia coli* isolated from samples of different biological and environmental sources. *Bangladesh Journal of Veterinary Medicine*. *5*(2), 25–32.

APPENDICES

Appendix 1: Melt Curve for Optimized *E. coli* strains

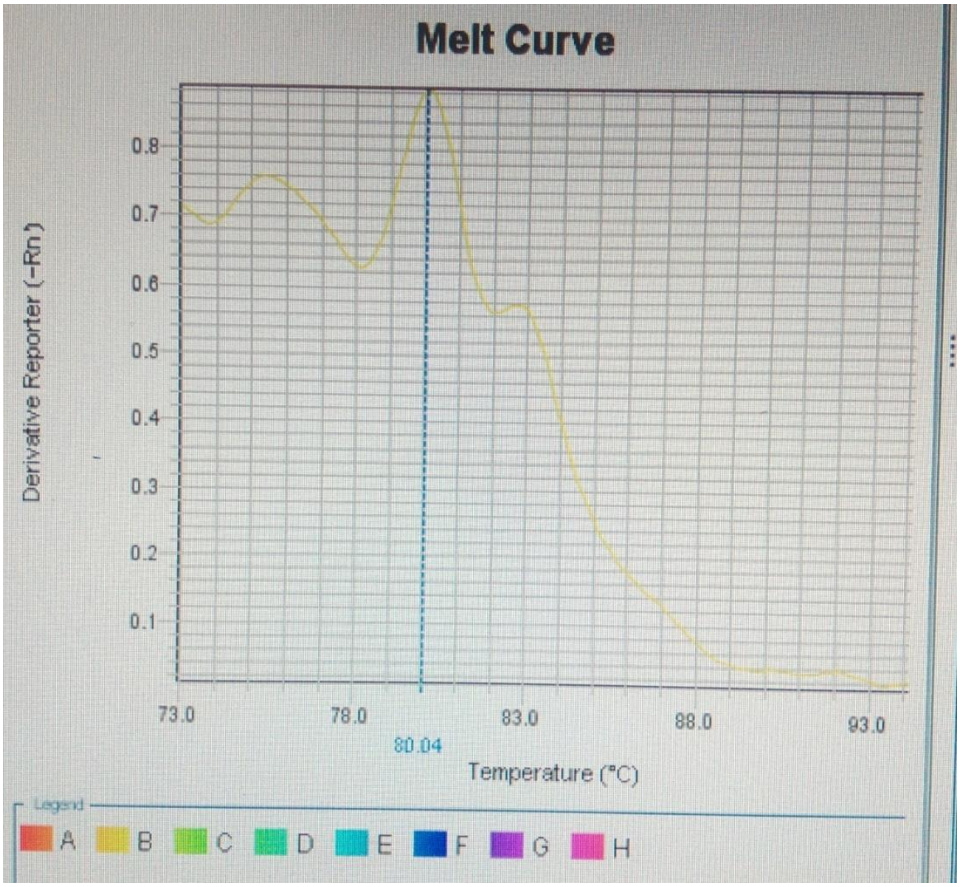


Fig. 22 Melt curve for Optimized *stIa*

The *y* axis (fluorescence) represents the negative derivative of fluorescence over temperature.

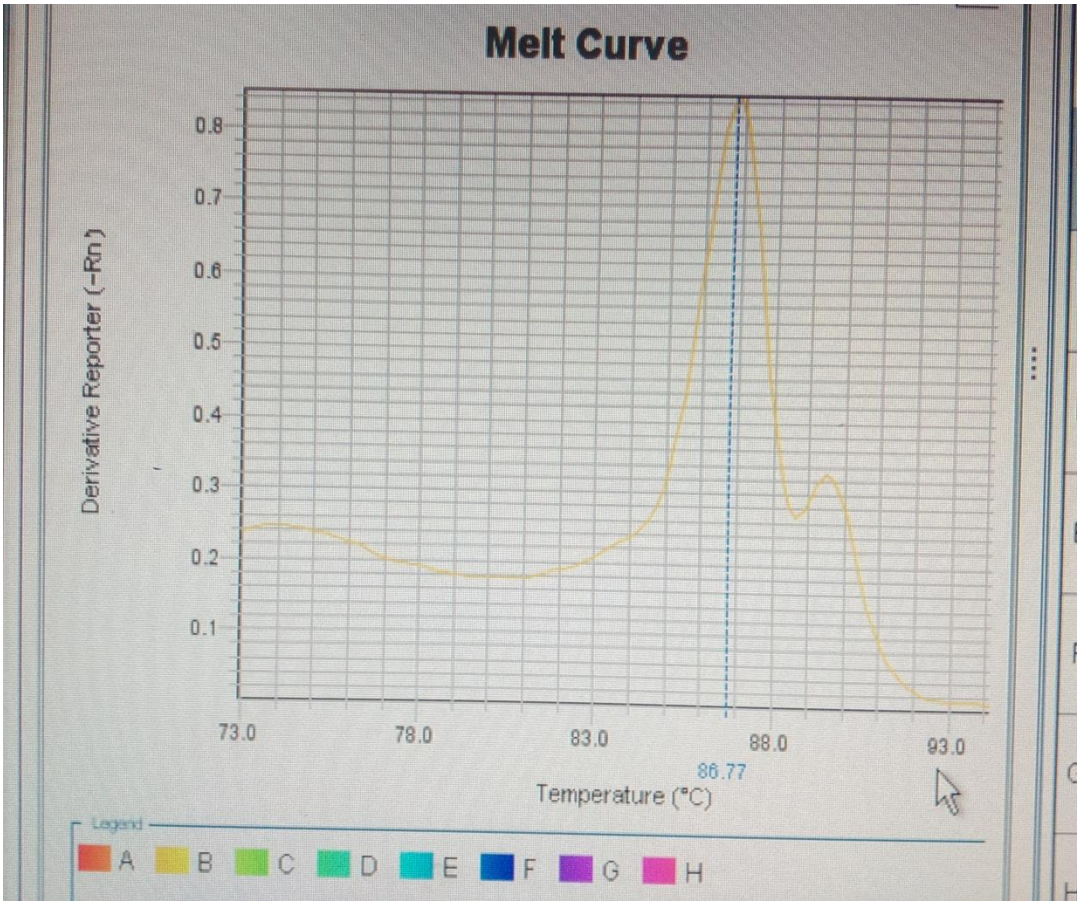


Fig. 23 Melt curve for optimized stx1 at Tm 87.0 ±0.23 and *stx2* at Tm 89.1 ± 0.34

The *y* axis (fluorescence) represents the negative derivative of fluorescence over temperature.

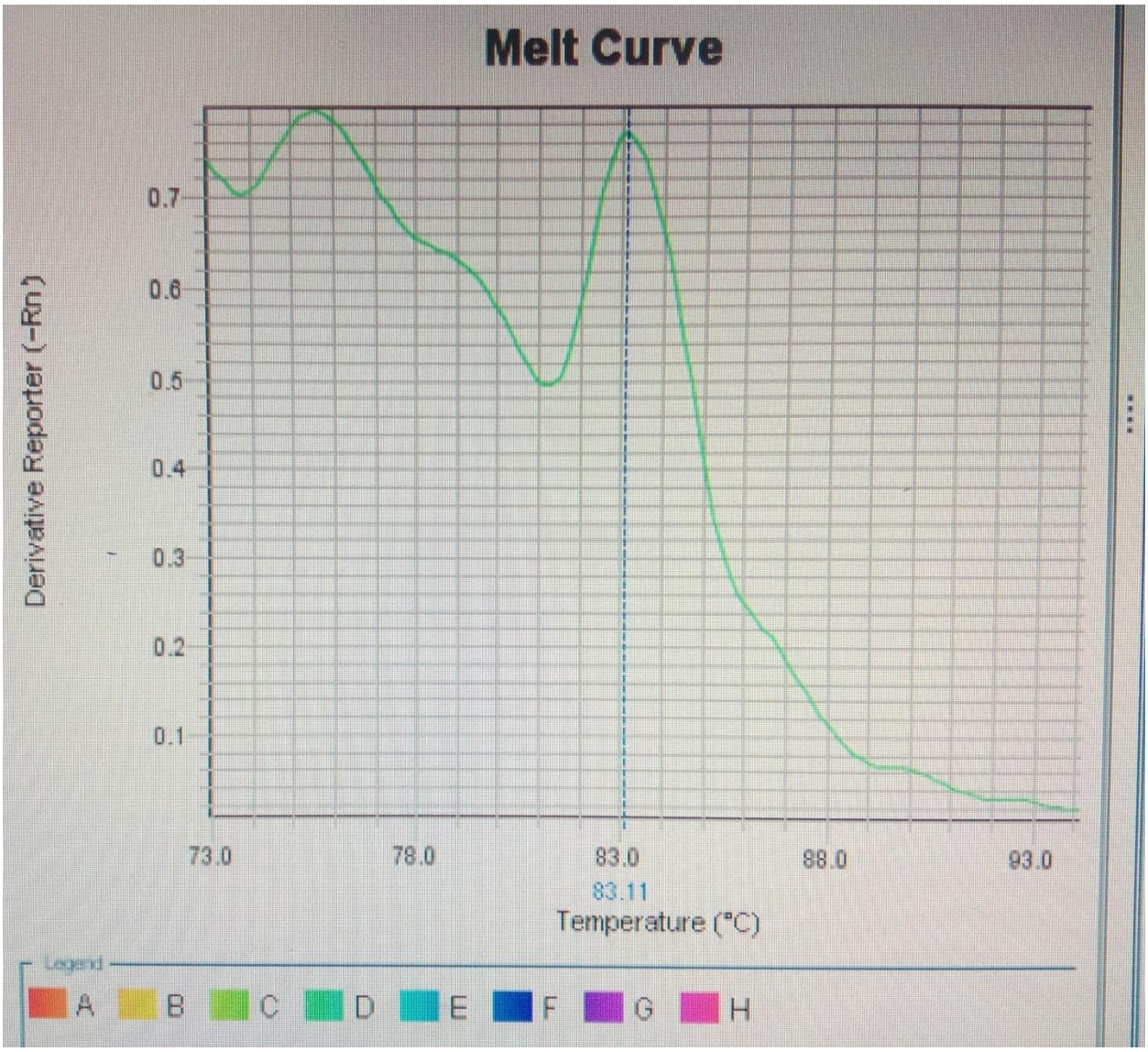


Fig. 24 Melt curve for optimized *eaeA* at Tm 83.3 ±0.32

The *y* axis (fluorescence) represents the negative derivative of fluorescence over temperature.

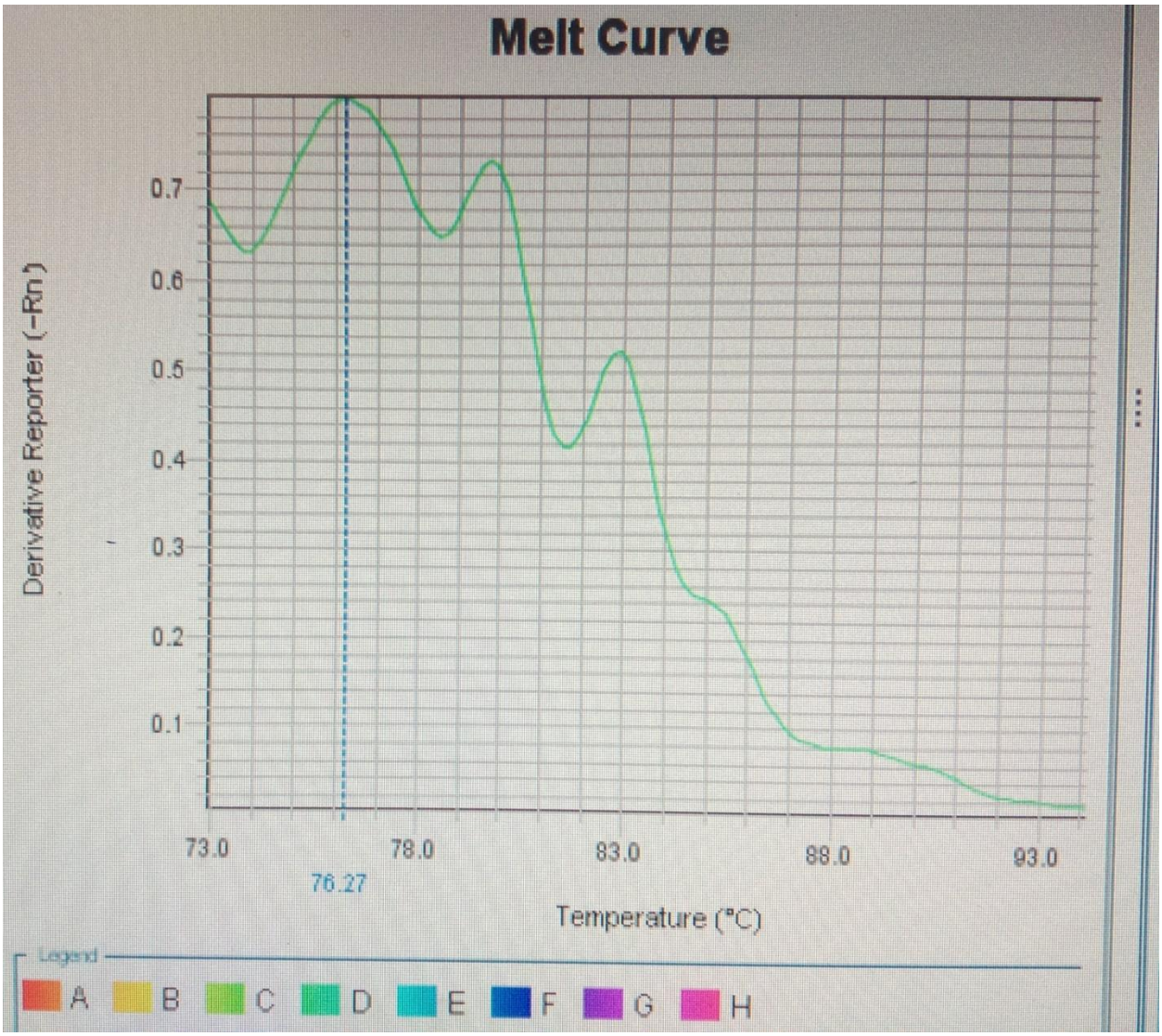


Fig. 25 Melt curve for optimized *ipaH at 77*±0.26, *stx2 at* Tm 89.1 ±0.34 and *eaeA* at Tm 83.4 ±0.08

The *y* axis (fluorescence) represents the negative derivative of fluorescence over temperature.

Appendix 2: *Wagashi* Pre-Processing Microbial Presence

Process	acid-adapted *E. coli*	non acid-adapted *E. coli*
Raw Milk + Inoculum	4.2	4.2
Pasteurisation	0	0
Curdling	0	0
Cooling	0	0
***Wagashi* Day 1**	0.2	0.4
Whey Day 1	0.4	0.6
***Wagashi* Day 2**	0.9	1.1
Whey Day 2	1.2	1.4
***Wagashi* Day 3**	2.1	2.7
Whey Day 3	2.9	3.1

Appendix 3: *Wagashi* Post-processing Microbial Presence

Process	acid-adapted *E. coli*	non acid-adapted *E. coli*
***Wagashi* + Whey**	5	5.8
Boil (72°C for 15 sec)	0	0
Cool (30°C) + Inoculum	4.2	4.2
***Wagashi* Day 1**	4.4	4.6
Whey Day 1	4.4	4.6
***Wagashi* Day 2**	4.7	4.9
Whey Day 2	4.9	5.1
Wagashi Day 3	5.2	5.4
Whey Day 3	5.5	5.7

Appendix 4: Analysis of E. coli survival in media of pH of 3.5

ANOVA Table for acid-adapted by time in media of pH 3.5

Source	***Sum of Squares***	***Df***	***Mean Square***	***F-Ratio***	***P-Value***
Between groups	18.044	4	4.511	24.52	0.0018
Within groups	0.92	5	0.184		
Total (Corr.)	18.964	9			

ANOVA Table for non-acid-adapted by time in media of pH 3.5

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	33.304	4	8.326	50.77	0.0003
Within groups	0.82	5	0.164		
Total (Corr.)	34.124	9			

Comparison of Means

95.0% confidence interval for mean of acid-adapted: 6.6625 +/- 1.10545 [5.55705, 7.76795]

95.0% confidence interval for mean of non-acid-adapted: 0.375 +/- 0.622021 [-0.247021, 0.997021]

95.0% confidence interval for the difference between the means

assuming equal variances: 6.2875 +/- 1.15051 [5.13699, 7.43801]

t test to compare means

Null hypothesis: mean1 = mean2

Alt. hypothesis: mean1 NE mean2

assuming equal variances: t = 11.7212 P-value = 1.26396E-8

Reject the null hypothesis for alpha = 0.05.

Appendix 5: Analysis of E. coli survival in media of pH of 4

ANOVA Table for acid-adapted by Time in media of pH 4

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	3.426	4	0.8565	28.55	0.0012
Within groups	0.15	5	0.03		
Total (Corr.)	3.576	9			

ANOVA Table for non-acid-adapted by Time in media of pH 4

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	20.9103	4	5.22758	15.32	0.0052
Within groups	1.7063	5	0.34126		
Total (Corr.)	22.6166	9			

Comparison of Means

95.0% confidence interval for mean of acid-adapted: 5.975 +/- 0.452976 [5.52202, 6.42798]

95.0% confidence interval for mean of non-acid-adapted: 1.295 +/- 0.659952 [0.635048, 1.95495]

95.0% confidence interval for the difference between the means

assuming equal variances: 4.68 +/- 0.726034 [3.95397, 5.40603]

t test to compare means

Null hypothesis: mean1 = mean2

Alt. hypothesis: mean1 NE mean2

assuming equal variances: t = 13.8253 P-value = 1.48963E-9

Reject the null hypothesis for alpha = 0.05.

Appendix 6: Analysis of *E. coli* survival at a temperature of 50 ℃

ANOVA Table for acid-adapted by Time at temperature of 50 ℃

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	0.4225	1	0.4225	33.80	0.0283
Within groups	0.025	2	0.0125		
Total (Corr.)	0.4475	3			

ANOVA Table for non-acid-adapted by Time at temperature of 50 ℃

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	17.2225	1	17.2225	1377.80	0.0007
Within groups	0.025	2	0.0125		
Total (Corr.)	17.2475	3			

Appendix 7: Analysis of *E. coli* survival at a temperature of 55 ℃

ANOVA Table for acid-adapted by Time at a temperature of 55 ℃

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	6.76	1	6.76	5.10	0.1524
Within groups	2.65	2	1.325		
Total (Corr.)	9.41	3			

ANOVA Table for non-acid-adapted by Time at a temperature of 55 ℃

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	21.6225	1	21.6225	345.96	0.0029
Within groups	0.125	2	0.0625		
Total (Corr.)	21.7475	3			

Appendix 8: Analysis of *E. coli* survival of E. coli in a media of 2% salt concentration

ANOVA Table for acid-adapted by Time in media of 2% salt concentration

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	10.1338	3	3.37792	19.17	0.0078
Within groups	0.705	4	0.17625		
Total (Corr.)	10.8388	7			

ANOVA Table for non-acid-adapted by Time in media of 2% salt concentration

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	28.3138	3	9.43792	2516.78	0.0000
Within groups	0.015	4	0.00375		
Total (Corr.)	28.3288	7			

Comparison of Means

95.0% confidence interval for mean of acid-adapted: 6.51667 +/- 1.07518 [5.44148, 7.59185]

95.0% confidence interval for mean of non-acid-adapted: 0.333333 +/- 0.286761 [0.0465721, 0.620095]

95.0% confidence interval for the difference between the means

assuming equal variances: 6.18333 +/- 0.964529 [5.2188, 7.14786]

t test to compare means

Null hypothesis: mean1 = mean2

Alt. hypothesis: mean1 NE mean2

assuming equal variances: t = 14.284 P-value = 5.58886E-8

Reject the null hypothesis for alpha = 0.05.

Appendix 9: Analysis of *E. coli* survival of *E. coli* in a media of 3% salt concentration

ANOVA Table for Acid-adapted by Time in media of 3% salt concentration

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	2.87	3	0.956667	2.92	0.1636
Within groups	1.31	4	0.3275		
Total (Corr.)	4.18	7			

ANOVA Table for non-acid-adapted by Time in media of 3% salt concentration

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	26.125	3	8.70833	68.30	0.0007
Within groups	0.51	4	0.1275		
Total (Corr.)	26.635	7			

Comparison of Means

95.0% confidence interval for mean of Acid-adapted: 5.38333 +/- 0.703724 [4.67961, 6.08706]

95.0% confidence interval for mean of non-acid-adapted: 0.55 +/- 0.916683 [-0.366683, 1.46668]

95.0% confidence interval for the difference between the means

assuming equal variances: 4.83333 +/- 1.0017 [3.83163, 5.83503]

t test to compare means

Null hypothesis: mean1 = mean2

Alt. hypothesis: mean1 NE mean2

assuming equal variances: t = 10.7511 P-value = 8.15335E-7

Reject the null hypothesis for alpha = 0.05.

Appendix 10: Analysis of E. coli survival of E. coli in a media of combined treatment of 3% salt concentration, pH 4 at a temperature of 50°C

ANOVA Table for acid-adapted by Time at combined treatment of temp. 50, salt 3%and pH4

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	30.37	4	7.5925	14.19	0.0061
Within groups	2.675	5	0.535		
Total (Corr.)	33.045	9			

ANOVA Table for non-acid-adapted by Time at combined treatment of temp. 50, salt 3%and pH4

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	29.574	4	7.3935	739.35	0.0000
Within groups	0.05	5	0.01		
Total (Corr.)	29.624	9			

Comparison of Means

95.0% confidence interval for mean of acid-adapted: 2.65 +/- 1.37074 [1.27926, 4.02074]

95.0% confidence interval for mean of non-acid-adapted: 1.26 +/- 1.29785 [-0.0378497, 2.55785]

95.0% confidence interval for the difference between the means

assuming equal variances: 1.39 +/- 1.75314 [-0.363138, 3.14314]

t test to compare means

Null hypothesis: mean1 = mean2

Alt. hypothesis: mean1 NE mean2

assuming equal variances: t = 1.66575 P-value = 0.113071

Do not reject the null hypothesis for alpha = 0.05.

Appendix 11: Shelf-life studies

ANOVA Table for Acid-adapted by Time

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	2.8575	5	0.5715	27.43	0.0005
Within groups	0.125	6	0.0208333		
Total (Corr.)	2.9825	11			

ANOVA Table for non-acid-adapted by Time

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	4.02417	5	0.804833	193.16	0.0000
Within groups	0.025	6	0.00416667		
Total (Corr.)	4.04917	11			

Comparison of Means

95.0% confidence interval for mean of Acid-adapted: 4.79 +/- 0.351182 [4.43882, 5.14118]

95.0% confidence interval for mean of non-acid-adapted: 5.12 +/- 0.375213 [4.74479, 5.49521]

95.0% confidence interval for the difference between the means

assuming equal variances: -0.33 +/- 0.47729 [-0.80729, 0.14729]

t test to compare means

Null hypothesis: mean1 = mean2

Alt. hypothesis: mean1 NE mean2

assuming equal variances: t = -1.45259 P-value = 0.163549

Do not reject the null hypothesis for alpha = 0.05.