UNIVERSITY FOR DEVELOPMENT STUDIES

DETECTION OF *LISTERIA* SPECIES AND *LISTERIA MONOCYTOGENES* IN RAW MILK AND MILK PRODUCTS IN THE NORTHERN REGION, GHANA

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DECLARATION

I do hereby declare that, except for references to the work of others that have been duly cited, this work is the result of my own original research under the supervision of Dr. James Owusu-Kwarteng and that this thesis either in whole or part has not been presented for another degree elsewhere.

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ABSTRACT

There is little information about the quality of the milk and its derived products either at farm level or at market level in Ghana. As a result it is important to investigate the quality and safety of milk and its derived products in order to improve the nutritional base of the increasing Ghanaian population and the marketing of the milk and its derived products. The purpose of this study was therefore to detect and determine the prevalence of *Listeria* species and *L. monocytonenes* in milk and milk products in the Northern Region of Ghana using different selective culture media and PCR-based techniques. Prior to the collection of samples of milk and milk products for bacteriological analysis, a questionnaire based survey was administered to 85 dairy farm owners and processors about factors that help in reducing the level of contamination of milk and its products by *Listeria* species. All the respondents indicated that cleaning/disinfection of cattle udder before milking is not commonly practice. The milk products commonly made by producers include *Nunu,* soft cheese, yoghurt and butter. Milk intended to be sold directly to consumers is boiled by the producers. All (100%) the cattle farmers feed their cattle by means of free range system. About 39.2% of dairy farmers regularly vaccinate their cattle against some of the common cattle diseases. A total of 163 samples of milk and milk products were purchased from producers, traders and vendors to examine the presence of *Listeria* species and *L. monocytogenes* by conventional isolation, colony PCR and enrichment PCR methods. The conventional method detected 34/163 (20.9%) and 38/163 (23.3%) *Listeria* species of the experimental samples based on growth on oxford and palcam selective media respectively. While colony PCR detected 21 *Listeria* contaminated samples, Enrichment PCR detected 22 *Listeria* contaminated samples. The prevalence of *L. monocytogenes* was 6.7% (11/163) in both colony and enrichment PCR methods. Among the studied samples of milk and milk products, soft cheese had the highest prevalence (33.3%) of *Listeria,* followed by raw milk (17.5%) and then spontaneously

fermented yoghurt (11.5%). *Listeria* species and *L. monocytogenes* were not detected in pasteurized milk on market, fried cheese and yoghurt produced with commercial starter culture. The efficiencies of oxford agar and palcam agar in this study were 91.1% and 87.7% respectively, however, there was no significant difference among the two media in the detection of *Listeria* species. Again, there was no significant difference between the enrichment PCR technique and the colony PCR (P >0.05). In conclusion, this study reveals that consumption of raw milk, soft cheese and spontaneously fermented milk could serve as potential risks of listeriosis in this region. The scenario warrants that milk is pasteurized and safety practices ensured during processing before consumption.

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DEDICATION

This work is dedicated to my wife, Esther and child, Divine Wun-Yiko who endured my absence during the course of my study and to all neonates and other susceptible individuals to listeriosis who might have lost their lives as a result of *Listeria* infections.

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LIST OF ABBREVIATIONS

BPW	Buffered peptone water
CAMP	Christie Atkins Munch-Petersen
CDC	Centre for Diseases Control
CFU	Colony forming unit
DNA	Deoxyribonucleic Acid
FAO	Food and Agriculture Organization
FDA	Food and Drugs Administration
GSS	Ghana Statistical Services
ISO	International Organization for Standards
KOH	Potassium Hydroxide
LLO	Listeriolysin O
MPI	Ministry for Primary Industries
PCR	Polymerase Chain Reaction
RTE	Ready to Eat
US	United States
USDA	United States Department of Agriculture
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTON

1.1 Background and Justification

Bacteria of the genus *Listeria* are gram positive, facultative anaerobic, catalase positive, oxidase negative, non spore forming (Robert and Greenwood, 2003; McLauchlin and Rees, 2009) and taxonomically, consist of six species which include *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, and *L. grayii* (Kasalica *et al.*, 2011; Zamani-Zadeh *et al.*, 2011). *L. monocytogenes* is a food-borne pathogen in humans and animals (Michael *et al.*, 2005; Cossart, 2011). The organism has the ability of growing at <0°C - 45°C usually under aerobic and facultative anaerobic conditions (McLauchlin and Rees, 2009). They grow optimally at neutral to slightly alkaline pH (McLauchlin and Rees, 2009).

Listeria is widely distributed in the environment and has been isolated by several researchers from water (Lyautey *et al.*, 2007, 2012), sewage (Luppi *et al.*, 1988) and processing plants (Chambel *et al.*, 2007) and from the faeces of healthy animals (Husu *et al.*, 1990). Some have isolated *Listeria* from foods such as vegetables (Sangeetha and Shubha, 2014), dairy products (Abrahao *et al.*, 2008), meat (Eruteya *et al.*, 2014), fish and sea-food (Wagner *et al.*, 2007; Jamali *et al.*, 2012), and frozen food products (Loncarevic *et al.*, 1997). The diffused nature of *Listeria* has the tendency of incapacitating the food industries in producing food free of *Listeria* (Morobe *et al.*, 2012).

The composition of milk makes it an important source of proteins, calcium and energy for infants and young children who have few alternative sources for these nutrients (Hempen *et al.*, 2004). A high standard of hygiene is required in milk production and processing; a truth recognized in most countries where milk was the first food to be the focus of modern food hygiene legislation (Adams and Moss, 2008). A milk product refers to any food that has been

processed from milk. Fresh milk remains the starting point for these milk products. The conversion of milk into milk products are mainly for preservation and the conservation of the milk nutrients (Adams and Moss, 2008).

The frequent contamination of the udder, teats, fur and skins of cows by *Listeria* from the environment makes the organism easily get into milk during milking (Hempen *et al*., 2004). Also, if the milking and processing procedures and the equipment used for milking, filtering, distributing, cooling, storing, and processing milk and milk products are not hygienic, they may equally serve as sources of *Listeria* contamination (Hempen *et al*., 2004). The individuals carrying out the milking of animals, handling the milk, selling the milk and processing the milk into products can contribute to the milk and milk products getting contaminated with *Listeria*.

Milk and milk products have been implicated as vehicles for *L. monocytogenes* transmission to humans. *L. monocytogenes* is the main cause of listeriosis in both humans and animals. Listeriosis is a rare but serious food-borne disease since it exhibits about 50% neonatal death rates, 20-30% mortality and 91% hospitalization in immunocompromised groups (Low and Donachie, 1997; Kathariou, 2002; Jemmi and Stephan, 2006). Listeriosis is usually characterized by gastroenteritis, meningoencephalitis, still birth, septicemia, premature birth and spontaneous abortions (Tano-Debrah *et al*., 2011) and is common among the immunosuppressive individuals (Rocourt and Cossart, 1997).

Special standardized protocols have been designed by regulatory agencies for the enrichment and isolation of *Listeria* from food. These isolation protocols include the International Standards Organization (ISO), US Food and Drug Administration (FDA) and US department of Agriculture (USDA) (Liu, 2008). The choice of a technique for isolation depends on the

food under test (Oxoid, 2006; Jamali *et al.,* 2012). Several nonselective enrichment broths exist for these protocols; however limited selective agars are available for detecting and isolating *Listeria* (Jamali *et al.,* 2012). Also the residence flora present in a given food source would dictate the type of enrichment to be used. All *Listeria* species are capable of hydrolyzing esculin and this trait is therefore used to distinguish between *Listeria* species and other bacteria on oxford agar (Encinas *et al*., 1999) and palcam agar (El Marrakchi *et al*., 2005). Previous studies conducted by different researchers had found oxford agar more superior than palcam agar (El Marrakchi *et al.,* 2005; Jamali *et al.,* 2012). Raw milk, minimally and high processed milk products were tested for the *Listeria* species. Test on sensitivity and specificity of the two media were examined.

The microbiological specification for food items including milk and milk products has therefore stipulated the absence of *L. monocytogenes* in 25 g of food samples (Roberts *et al*., 1993; Robert and Greenwood, 2003). Thus various culture and PCR methods have been adopted to examine food samples to ensure they conform to this regulation. Confirmation of *Listeria* from isolates by PCR procedure is straightforward as inhibitory substances in the food samples are removed during the culturing process (Liu, 2008) just that it is laborious (Hudson *et al*., 2001). The pre-enrichment and enrichment of foods prior to PCR or plating on selective agar media resuscitate injured bacteria (Roberts *et al*., 1993) and also increases the concentration of bacteria to detectable levels (Gracias and McKillip, 2004).

High prevalence of *Listeria* species in raw milk and milk products in different countries has been observed (Gaya *et al*., 1998; Mugampoza *et al*., 2011; Gebretsadik *et al*., 2011; Hassan *et al*., 2000; Ning *et al*., 2013). Raw milk is reported to have high nutritional qualities and a health benefit compared to pasteurized and powdered milk, and is sometimes packaged as ready –to- eat product in certain countries (Venegas *et al*., 2009). In Ghana and other parts of West Africa, fresh milk is routinely consumed by the Fulani tribesmen or processed into

various fermented and non-fermented products (Obodai and Dodd, 2006; Akabanda *et al*., 2010, 2013, 2014). Recent studies suggest the occurrence of *L. monocytogenes* in some foods including milk and milk products in the southern parts of Ghana (Appiah, 2012; Dobge, 2010). In general, milk and milk products are easily contaminated, and may comprise a major source of infection (Swaminathan and Gerner-Smidt, 2007). Although the high risks associated with consuming milk and milk products are well-known worldwide, there is little or no information on the prevalence of *Listeria* species in milk in the Northern region of Ghana.

1.2 Objectives

1.2.1 Main Objective

This study is aimed at determining the prevalence of *Listeria* species and *Listeria monocytogenes* in milk and milk products in the Northern Region of Ghana.

1.2.2 Specific Objectives

1. To assess the management practices in dairy farming and the processing of milk into various products in the Northern region of Ghana.
2. To isolate and identify *Listeria* species contaminating fresh milk and milk products
3. To determine the efficiency of detecting the *Listeria* species using different selective culture media.
4. To compare the effectiveness of Enrichment PCR and Colony PCR in detecting *Listeria* species and *L. monocytogenes* in raw milk and milk products.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The *Listeria* species

Listeria is one of the genera of bacteria comprising of six species which include *L. monocytogenes, L. innocua, L. ivanovii, L. welshimeri, L. murrayi* and *L. grayi* (Zamani-Zadeh *et al.,* 2011). DNA homology values, 16S rRNA sequencing homology, chemotaxonomic properties and multilocus analysis support the evidence of the six species of *Listeria* (Rocourt, 1999). The six species have the same genetic homology and this helps in explaining their similar phenotypic traits (Sallen *et al.,* 1996; Rocourt, 1999). The genus *Listeria* was named after Lord Lister and received its current name in 1940 (Wagner and Mcluachlin, 2008; Mcluachlin and Rees, 2009). The Genus *Listeria* was classified in the family Listeraceae in 2004 (Mcluachlin and Rees, 2009). They belongs to the group of bacteria with low G + C DNA content (55%) (Rocourt, 1999). *Listeria* measures 0.5µm in diameter and 1 - 2 µm in length (Rocourt, 1999; Yeh, 2004) and have rounded ends (Rocourt, 1999). On artificial media, *Listeria* colonies after 24-48 hours are 0.5-1.5 mm in diameter, low convex with a smooth surface and entire margin, and nonpigmented with crystalline central appearance. Older cultures (3-7days) have opaque appearance and sometimes develop sunken center and the *Listeria* in such cultures are large with a diameter of about 3-5 mm (Wagner and Mcluachlin, 2008). Microscopically, they appear as rods and are distributed individually as short chains in the form of the letters V and Y. They can assume coccoid form and may be mistaken for stretococci (Rocourt, 1999; Kasalica *et al.,* 2011). Some of the cells lose their ability to retain gram stain at older age (Rocourt, 1999; Wagner and Mcluachlin, 2008) and may be mistaken for *Haemophilus* (Rocourt, 1999).

Genomic analysis of the genus *Listeria* put the species into three main groups comprising *L. monocytogenes, L. innocua,* and *L. welshimeri* as the first group, *L. invanovii* and *L. seeligeri*

as the second group and finally *L. grayi* as the third group (Michael *et al*., 2005). *L. grayi* represent the original ancestor of the genus *Listeria* (Wagner and Mcluachlin, 2008).

With the exception of *L. monocytogenes* and *L. invanovii* the rest of the *Listeria* species are not pathogenic in nature (Cossart, 2011).

2.2 Factors that influence the growth of *Listeria*

Members of *Listeria* genus have petrichous flagella and hence motile when cultured at a temperature of 20-25°C but are nonmotile at 37°C (Kasalica *et al.*, 2011). The synthesis of flagella for motility in *Listeria* is temperature and catabolite regulated (Vatanyoopaisarn *et al*., 2000; Grundling *et al*., 2004). Generally, *Listeria* species are psychrophilic, surviving at a temperature below 0°C (Wagner and Mcluachin, 2008). *Listeria* grows well in media with pH levels between 4.4–9.4 and water activity above 0.92 (Aygun and Pehlivanlar, 2006). They grow optimally at low salt concentration and slowly at high salt concentration (Zamani-Zadeh *et al*., 2011). Depending on factors such as moisture content, storage temperature, presence of starter cultures, *L. monocytogenes* can grow up to a pH of 4.1. *Listeria* grows optimally at low salt concentration and very slowly at high salt concentration (Zamani-Zadeh *et al*., 2011). *Listeria* species requires fewer nutrients such as biotin, riboflavin, thiamine, thioctic acid, and amino acids (e.g., cysteine, glutamine, isoleucine, leucine, and valine) for optimal growth (Wagner and Mcluachlin, 2008). Carbohydrates (example, glucose) are also essential for *Listeria* multiplication. They produced acid as a by-product in carbohydrates (Wagner and Mcluachlin, 2008). Artificially, *Listeria* can grow under both aerobic and anaerobic conditions on nonselective media such as tryptone soy broth or brain-heart infusion (BHI) broth (Wagner and Mcluachlin, 2008). It has been observed that tumbling motility of *Listeria* occurs when cultures are maintained between 20 and 25°C in any of these nonselective media (Wagner and Mcluachlin, 2008). A wide range of selective media such as palcam agar and

oxford agar are now available for the isolation of *Listeria* species (Wagner and Mcluachlin, 2008). Gysemans *et al.* (2007); Vermeulen *et al.* (2007), indicated that *L. monocytogenes* failed to grow at water activity (a_w) < 0.930, pH < 4.3 or a total acetic acid concentration > 0.4% when incubated at 7°C in nutrient broth. In their study different combinations of environmental factors including pH 5.0–6.0, water activity (a_w) 0.960–0.990, and acetic acid concentration 0–0.8% (w/w) were employed. Thus it is important for the food industries to determine environmental conditions responsible for maintaining the freshness of food while keeping microbial contamination at a minimum level, in order to ensure that manufactured foods are safe for human consumption (Wagner and Mcluachlin, 2008).

2.3 Distribution of *Listeria* in the environment

2.3.1 Distribution in water and sludge

Bacteriological analyses have previously shown that unpolluted water rarely contains *Listeria* as compared to polluted water. Luppi *et al.* (1988) isolated 11 *Listeria* strains (including one *L. monocytogenes*, two *L. seeligeri,* one *L. welshimeri* and seven *L. innocua*) from 50 river water samples (22.0%); 15 *Listeria* strains (which includes 4 *L. monocytogenes,* 11 *L. innocua*) from 80 surface water samples (18.8%); 1 *Listeria* strain (*L. innocua*) representing 1.0% of 98 samples from ground water; 14 strains (8 *L. monocytogenes*, 5 *L. innocua,* 1 *L. seeligeri*) representing 42.4% of 33 samples of urban sewage. Lyautey *et al.* (2012) isolated 187 *L. monocytogenenes* from 45 positive water samples out of a total of 126 surface water samples. In another related work, Lyautey *et al.* (2007) isolated 2,826 *Listeria* strains of which 75 *L. monocytogenes* were isolated from 200 (64%) out of 314 surface water samples. Frances *et al.* (1991) examined 30 samples of surface water and *Listeria* species were isolated from 8 samples (27%) and were identified as 6 *L. seeligeri,* 1 *L. innocua* and 1 *L. welshimeri.* Taherkhani *et al.* (2013) examined 66 samples of influent, effluent and river water for positive samples of *Listeria* species and *L. monocytogenes.* The total number of positive

samples for *Listeria* species and *L. monocytogenes* were 20 (30.30%) and 18 (27.27%) respectively and the prevalence in samples collected from influent, effluent and river water were 5 (83.83%), 4 (66.66%) and 11 (20.73%) for *Listeria* species, and 5 (83.83%), 3 (50%) and 10 (18%) for *L. monocytogenes* respectively.

2.3.2 Distribution in food processing plants

Food processing environments are important points of entry for *Listeria* species into the food chain. The food processing environments include surfaces of food processing benches, equipment, floors and machinery. The *Listeria* species easily adheres to these surfaces and subsequently grows into biofilm matrix with increase resistance to adverse conditions (Blackmann and Frank, 1996). In the food and dairy industries, *Listeria* has been isolated from biofilms where they adhere to and colonise moist areas and equipment thereby posing a risk of recontamination of the finished product (Lou and Yousef, 1999; Holah *et al*., 2002). Chae and Schraft (2000) grew *L. monocytogenes* on glass slides in to biofilms and realized that all 13 strains of *L. monocytogenes* were found attached to the glass slides and formed biofilms but there was difference in the rate of biofilm formation among the various strains of *L. monocytogenes* suggesting that some strains of *L. monocytogenes* can easily grow into biofilm than others. Chambel *et al.* (2007) reported that eight dairies located in two regions of Portugal were screened along the production cycle to determine the presence and distribution of *Listeria* species, and three dairies in each region were positive for the presence of *Listeria* and 213 *Listeria* isolates were obtained, comprising of 85 *L. monocytogenes,* 88 *L. innocua*, 39 *L. seeligeri*, and one *L. invanovii*. Additionally, Thevenot *et al.* (2005) observed that the occurrence of *Listeria monocytogenes* on surfaces of processing plants before and during processing varies with low percentage being recorded before the beginning of processing.

2.4 *Listeria* in food

2.4.1 Distribution in meat

The biological value of meat cannot be overemphasized. Meat serves as a major source of protein and it is very rich in iron, zinc and vitamins such as vitamin A and B (Eldaly *et al.* (2013). A researched carried out by Vitas *et al.* (2004) showed the occurrence of 76.3% *Listeria* species and 36.1% *L. monocytogenes* in raw poultry samples in Northern Spain. Kosek-Paszkowska *et al.* (2005) studied the occurrence of *Listeria* species in raw poultry meat and poultry meat products and isolated *Listeria* species from 36 samples of raw chicken parts (51.4%) and 7 samples of poultry minced meat (30.4%), and also realized 14% prevalence of *L. monocytogenes.* Dimic *et al.* (2010) studied about the presence of *Listeria* species in fresh meats (beef, chicken and pork) and identified the bacteria in 82.7% (24) of 29 analyzed samples. These included 7 *L. innocua,* 8 *L. monocytogenes* and 9 *L. welshimeri.* Eldaly *et al.* (2013) investigated the prevalence of *L. monocytogenes* in slaughtered carcasses of buffalo, sheep and cattle in Egypt which has passed post-mortem test and hence fit to be consumed by humans and noticed that *L. monocytogenes* was isolated from 14 (6.7%) buffalo samples, 23 (10.95%) sheep samples and 11 (4.8%) cattle samples respectively. Eruteya *et al.* (2014) conducted a bacteriological analysis on raw cow and goat meat in Port Harcout, Nigeria that led to the identification of 310 *Listeria* strains (4 *L. monocytogenes,* 20 *L. innocua,* 72 *L. seeligeri,* 4 *L. invanovi,* 71 *L. grayi* and 139 *L. welshimeri*) in 81 (33.75%) meat samples out of a total of 240 meat samples. In an earlier analysis, Luppi *et al.* (1988) identified 13 *Listeria* strains (nine *L. monocytogenes* and four *L. innocua*) in 113 meat samples. Capita *et al.* (2001) noted that the percentages of stored purchased fresh chicken carcasses in Brazil contaminated with *Listeria* species were *L. monocytogenes* (95%), *L. innocua* (32%), *L. welshimeri* (66%), *L. seeligeri* (7%), *L. grayi* (4%) and *L. ivanovii* (2%).

2.4.2 Distribution in raw milk and dairy products

Milk refers to the fluid secreted by mammals for the nourishment of their young. According to the MPI, raw milk is defined as "milk (secreted by mammals and used as food by human beings) that has not been subjected to any processing intended to alter the quality or composition characteristics of the milk." (MPI, 2013). There are several animals used to produce milk for human consumption, but the milk produce by cow is by far the most important in commercial terms. The main components of milk include water, fat, protein and lactose. The actual composition of milk varies between species, for example, the human milk has lower protein but higher lactose levels than cow's milk (Adams and Moss, 2008). Milk has high water activity, reasonable pH (6.4–6.6) and large supply of nutrients making milk an excellent medium for microbial growth including *Listeria* (Adams and Moss, 2008). As a result a high standard of hygiene is required in milk production and processing to minimize or totally eradicate microbial growth. Fresh milk refers to raw and pasteurized milk and these remains the starting point for a number of other milk products (Adams and Moss, 2008). These food products are the upgraded version of the fresh milk. They include Yoghurt, cheese, dried milk, sweetened condensed milk, pasteurized milk, evaporated milk and cream (Adams and Moss, 2008).

The nutritious nature of milk and milk products makes them prone to housing a number of microorganisms thereby serving as a medium for the growth of the organisms. The hygienic conditions during milking, handling and storage also makes milk products such as fermented milk prone to contamination by pathogenic organisms (Dalu and Feresu, 1996). Faecal and environmental contamination during milking, storage and transport, infected dairy animals and silage quality could serve as sources of *Listeria* in raw milk (Bemrah *et al.,* 1998). *L. monocytogenes* is quite resistant to heat and thus post-pasteurization storage at refrigeration temperature may allow growth of the organism (Dalton *et al.,* 1997). However, it is important

to note that the bacteriocins produced by lactic acid bacteria in some milk products such as yoghurt, suppresses the growth of pathogenic organisms (Jayamanne and Samarajeewa, 2001; Benkerrom *et al*., 2003). Stanczak *et al*. (1997) indicated that the micro flora of fermented milk products increased the death rate of *L. monocytogenes* especially when the products are stored at 20°C. Several studies have being conducted to screen milk and dairy products for the presence of *Listeria* species. Holko *et al.* (2001) detected *Listeria monocytogenes* in 18 positive samples of milk and dairy products. Waak *et al.* (2002) reported 19.6% *L. monocytogenes* and 8.5% *L. innocua* in raw whole milk from dairy silos. Navratilova *et al.* (2004) isolated *L. monocytogenes* from 15 samples out of a total of 389 raw milk samples and 1 sample out of a total of 20 pasteurized milk samples. The study carried out by Ahrabi *et al*. (1998) showed an incidence of 5% *Listeria* and none *L. monocytogenes* in pasteurized milk. Vitas *et al.* (2004) reported 8.1% positive samples of soft cheese in their study conducted in Northern Spain. Studies conducted by Abrahao *et al.* (2008) showed that 11 (12.20%) cheese samples from a total of 90 cheese samples were positive for *Listeria* species; *L. monocytogenes* was isolated from 6 samples (6.70%) and *L. innocua,* from five samples (5.50%). Research conducted by Gallegos *et al.* (2008) indicated that the frequency of *Listeria* species isolated from milk was 3.4% (15/440) and *L. monocytogenes* isolates was 9.2 % (42/440). The same researchers indicated that the frequency in cheeses for *L. monocytogenes* was 8.2% (47/573), *L. ivanovii* was 5.6% (32/573) and for the other *Listeria* species was 5.6% (32/573). Mugampoza *et al.* (2011) showed that *Listeria* species were detected in 60% of bulked raw milk, 30% of locally processed yogurt and 15% of Bongo, a traditionally fermented dairy product. Al-Ashmawy *et al.* (2014) revealed that 36% (72/200) of dairy products were positive for *Listeria monocytogenes* when a conventional biochemical test was employed.

Several other studies have reported that the incidence of *Listeria* species in raw milk samples varied from 0.76% to 44.4% in India (Shantha and Gopal, 2014), Turkey (Atil *et al*., 2011), Portugal (Kongo *et al*., 2006), Colombia (Gallegos *et al.*, 2008), Iran (Shamloo *et al.*, 2014), Spain (Vilar *et al*., 2007), Iraq (Alubaidy *et al*., 2013), India (Nayak *et al.*, 2015; Sreeja *et al.*, 2016), Syria (Al-Mariri *et al.*, 2013), and Ireland (Kells and Gilmour, 2004). That of *L. monocytogenes* incidence varied from 0.36 to 41.60 in China (Ning *et al*., 2013), Sweden (Waak *et al.*, 2002), Latvia (Konosonoka *et al.*, 2012), Czech Republic (Navratilova *et al.*, 2004), Iran (Jami *et al.*, 2010; Jamali *et al.*, 2013; Jamali and Radmehr, 2013), Turkey (Aygun and Pehlivanlar, 2006), Colombia (Gallegos *et al.*, 2008), Sri Lanka (Jayamanne and Samarajeewa, 2001), and Syria (Al-Mariri *et al.*, 2013). Numerous studies regarding milk products contamination by *Listeria* have been reported all over the world (Moura *et al*., 1993; Awaisheh, 2009; Arslan and Ozdemir, 2008; Akman *et al.*, 2004; Abrahao *et al.*, 2008; Pintado *et al*., 2004; Arrese and Izaga, 2012; Rahimi *et al.*, 2012; Shamloo *et al.*, 2014; Nayak *et al.*, 2015). Most studies all over the world have concentrated much on the incidence of *L. monocytogenes* because of its pathogenic nature. Hence, *L. monocytogenes* have been isolated from milk product worldwide (Rahimi *et al.*, 2012; Abrahao *et al.*, 2008; Shamloo *et al.*, 2014; Alubaidy *et al*., 2013; Arrese and Izaga, 2012; AL-Shamary, 2010; Awaisheh, 2009; Gelbicova and Karpiskova, 2009; Kevenk and Gulel, 2015).

Also, studies on milk and milk products in Africa have indicated that *Listeria* species have been isolated from samples of milk and milk products (Hempen *et al*., 2004; El-Shenawy, 2011; Mugampoza *et al*., 2011; Yakubu *et al*., 2012; Moshoeshoe and Oliver, 2012; Seyoum *et al*., 2015; Garedew *et al.*, 2015). In addition, *L. monocytogenes* contaminating milk and milk products in some parts of Africa has been reported by some authors (Molla *et al.*, 2004; El-Shenawy, 2011; Moshoeshoe and Oliver, 2012; Yakubu *et al*., 2012; El Marnissi *et al.*,

2013; AL-Ashmawy *et al.*, 2014; Seyoum *et al.*, 2015; Garedew *et al.*, 2015; Meshref *et al.*, 2015; Dabash *et al*, 2016).

Furthermore, a study undertaken by Tano-Debrah *et al.* (2011) in Accra, Ghana, to determine the presence and concentration of *L. monocytogenes* in different milk samples and other RTE food samples indicated a high prevalence of *L. monocytogenes* in milk samples. Summarized data for the prevalence of *Listeria* and *L. monocytogenes* in raw milk and milk products are presented in Table 2.1 and 2.2

Table 2.1: Prevalence of *Listeria* in Raw milk

Country	Number of samples	Method used	Prevalence (%)	References
Turkey	106	PCR	2 (1.19)	Atil *et al.*, 2011
Iran	91	PCR	5 (5.49)	Shamloo *et al.*, 2014
Portugal	105	PCR	2 (1.90)	Kongo *et al.*, 2006
India	50	PCR	8 (16.00)	Nayak *et al.*, 2015
Colombia	440	PCR	15 (3.40)	Gallegos *et al.*, 2008
Ireland	NA	PCR	NA (44.4)	Kells and Gilmour
Iraq	55	PCR	5 (9.00)	Alubaidy *et al.*, 2013
India	130	PCR	1 (0.76)	Shantha and Gopal, 2014
Syria	766	PCR	84(10.96%)	Al-Mariri *et al.*,2013
India	12	Culture	2 (16.66)	Sreeja *et al.*, 2016
Spain	98	NA	6 (6.1)	Vilar *et al.*, 2007
India	104	Culture	14 (13.46)	Saha *et al.*, 2015
Egypt	75	PCR	5 (6.66)	Dabash *et al*, 2016
Ethiopia	443	Culture	25 (5.60)	Seyoum *et al.*, 2015
Nigeria	192	Culture	17 (22.40)	Yakubu *et al.*, 2012
Morocco	288	Culture	17 (5.90)	El Marnissi *et al.*, 2013

NA: Not Available

Table 2.2: Prevalence of *Listeria* species in milk product

Country	Number	Method used	Product type	Prevalence (%)	References
Jordan	120	PCR	Milk, boiled cheese, soft cheeses, yoghurt, Labaney, Liquid Jameed, Shanineh, Ice cream	18 (15.00)	Awaisheh, 2009
Iran	201	PCR	Ice cream, Doogh, Yoghurt, Butter, Cream, Traditional cheese, Kashk, Fereni	16 (7.96)	Shamloo *et al.*, 2014
Iran	290	Culture	Ice cream, Cheese, Butter, Kashk	21 (7.2)	Rahimi *et al.*, 2012
Turkey	142	Culture	Cheese	47 (33.1)	Arslan and Ozdemir, 2008
Turkey	58	Culture	Ice cream	24 (41.4)	Akman *et al.*, 2004
Brazil	90	Culture	Cheese	11 (12.20)	Abrahao *et al.*, 2008
Portugal	63	Culture	soft cheeses	47 (75.00)	Pintado *et al.*, 2004
India	50	PCR	Ice cream, Milkshake, Fruit Salad	2 (4.00)	Nayak *et al.*, 2015
Brazil	220	Culture	Pasteurized milk	2 (FI0.90)	Moura *et al.*, 1993
Turkey	51	Culture	Cheese	5 (9.80)	Arrese and Izaga,2012
India	80	PCR	Ice cream	3 (3.70)	Moharram *et al.*, 2007

NA: Not Available

2.4.3 Distribution in ready-to-eat foods

Ready-to-eat foods ranges from preprocessed fish, meat, and vegetables that can be consumed without further cooking to process or cook food. Several ready-to-eat foods contain 2-5% NaCl and are usually stored at low temperature (Liu, 2008). *Listeria* species continue to grow even during the storage period (Liu, 2008).

Soultos *et al.* (2014) undertook a study on the prevalence of *L. monocytogenes* in 132 ready-to-eat seafood samples in Thessaloniki (Northern Greece), and they found *Listeria* species in 11 (8.3%) of the samples, with 8 (6.1%) yielding *L. monocytogenes* and only one sample (smoked mackerel) of the 8 highly contaminated. PCR analysis revealed three serotypes of *L. monocytogenes* strains isolated belonging to serogroup 1 (1/2a, 3a), 3 (1/2b, 3b, 7), 4 (4b, 4d, 4e) respectively. Gusman *et al.* (2014) also reported that of the 912 RTE food products (cooked meals, sandwiches and frozen foods) from Vojvodina province, Serbia, 18 (1.97) were positive for *L. monocytogenes* with cooked meals being highly contaminated. All the positive samples showed a high level of contamination of *L. monocytogenes* above 100 cfu/g food. El-shenawy *et al.* (2011) examined 576 street vended ready-to-eat food samples in Egypt (including poultry products, dairy products, meat products, plant products and seafood products) for the presence of *Listeria* species. Out of these number, 137 samples representing 24% were found to contain *Listeria* species, of which 78 (57%) were found to be contaminated with *L. monocytogenes* and 54 (39%) samples contained *L. innocua.* The other species of *Listeria* were found to be in lower frequency. Zhou and Jiao (2006) analyzed 844 ready-to-eat food samples (beef, chicken, liver, drumstick, pig tongue, pork and goose) purchased from some selected retail markets in China and 21 samples were contaminated with *L. monocytogenes*. Wagner *et al.* (2007) conducted an examination on 946 ready-to-eat food samples acquired from retail markets in Vienna, Austria and isolated 124 (13.1%) *Listeria* species and 45 (4.8%) *L. monocytogenes*. The isolates including 19.4% RTE fish and seafood, 5.5% soft cheese, 4.9% RTE raw meat sausages, and 6.3% cooked meat/pates products were positive for *L. monocytogenes.* Jamali *et al.* (2012) reported that of the 396 Ready-to-eat food samples, 71 (17.9%) samples contained *Listeria* species, 45 (11.4%) of which were identified to be *L. monocytogenes*.

2.4.4 Distribution in vegetables and fruits

The nutrient composition of vegetables and cut fruits serves as an ideal medium for the maintenance and growth of *Listeria* (Liu, 2008). A number of essential nutrients require by humans, such as vitamins (C, folate and pro-vitamin A), minerals (Potassium, Calcium and Magnesium), dietary fibre and phytochemicals (such as phenolics, flavonoids and carotenoids) for optimal nutrition, health and well-being are present in vegetables and fruits (Liu, 2013). Vegetable is therefore an indispensable food to both humans and *Listeria.* In 1979 the incidence of food-borne listeriosis (involving 23 patients) was reported in Boston Hospital where vegetables contaminated with *L. monocytogenes* 4b strain was responsible (Ho *et al.*, 1986). Schlech *et al*. (1983) indicated that in 1981 another epidemic of food-borne listeriosis occurred in the Maritime province, Canada where Coleslaw was implicated. Sangeetha and Shubha, (2014) reported that out of 165 samples of vegetables marketed in Mysore, Karnataka, India, examined for *Listeria* species, 3 samples were contaminated with *Listeria* species and in which *L. innocua* and *L. seeligeri* were confirmed. Dogbe (2010) examined fresh cabbage and ready-to-eat coleslaw for *L. monocytogenes* and reported prevalence rates of 95.8% and 80.1% respectively.

2.5 Prevalence of *Listeria* in animals

The widely spread nature of *Listeria* due to its ability to survive in polluted water, soil, vegetation, birds body, animal hair and foods makes animal feed such as silage and grasses, water and other foods, highly susceptible to the contamination of *Listeria* species hence the acquisition of the organism by animals (Liu, 2008; Konosonoka *et al.*, 2012).

Nightingale *et al.* (2004) conducted a study on the prevalence of *L. monocytogenes* in cattle, sheep and goat in 52 ruminants' farms (in New York) and realized 20.1% prevalence of *L. monocytogenes*. Gudmundsdottir *et al.* (2004) reported that 20 isolates of *L. monocytogenes*

were isolated from five confirmed and four suspected occurrences of listeriosis in horses in Iceland. Faeces from horses with severe signs of listeriosis had a very high load of *L. monocytogenes*. Kalorey *et al.* (2006) examined 50 faeces samples from wild animals (including one bird and six different mammals) and found 8 (16%) faecal samples with *L. monocytogenes.* Atil *et al.* (2011) isolated 46 *Listeria* species from 719 samples obtained from farmhouses of cattle and sheep, and cheese manufacturers from Elazig province in eastern Turkey. Konosonoka *et al.* (2012) investigated the incidence of *Listeria* species in Dairy cows feed in two different farms (including organic dairy farm and conventional dairy farm) in Lativia and noticed that 44.4% (24/54) and 18.3% (14/54) of *Listeria* species were isolated from organic dairy farm and conventional dairy farm respectively. Seifi (2012) conducted an evaluation on the prevalence of *L. monocytogenes* in Iranian broiler flocks and reported 8.97% positive samples. Forty four (44) out of a total of 490 broiler flocks were found to have been contaminated with the *L. monocytogenes*. Lawan *et al.* (2013) undertook a cross sectional research on the occurrence of *Listeria* species in ruminants in eastern Nigeria and reported that among a total of 300 faecal samples from cattle, sheep and goat, *Listeria* species were isolated from forty (13%) faecal samples (15 samples out of the 40 positive samples were isolated from cattle, 16 samples were isolated from sheep and 9 samples were isolated from goat). A related study conducted by Yadav *et al.* (2011) at Baroda zoo, Gujarat state, India, found that 5.4% (3/56) faecal samples from healthy animals were positive for *Listeria* species, of these samples, 1.8% (1/56) was identified to be *L. monocytogenes* and 3.6% (2/56) was *L. innocua.* Raorane *et al.* (2014) examined 215 samples (vaginal swabs, blood, milk, faeces, soil and floor swabs) from domestic animals and surroundings of cattle and pig farms which resulted in the isolation of 27 *Listeria* species. These included 11 *L. monocytogenes,* 2 *L. ivanovii,* 11 *L. innocua,* 1 *L. welshimeri,* and 2 *L. seeligeri*. From the above studies, it is clear that *Listeria* is found in animals.

2.6 Transmission routes of *Listeria* and infections in humans

There have not been any known occurrences of human to human transmission of *Listeria*, except that of the mother to fetus transmission (Marley, 2013). The consumption of contaminated food remains the most exclusive means of getting infected by *Listeria* (Bennet *et al*., 2000), however *Listeria* can be transmitted from transplacental following maternal bacteremia and ascending spread from vaginal colonization (i.e. mother to foetus), and through hospital acquired infections such as malignancy, organ transplants, immunosuppressive therapy, infection with the human immunodeficiency virus (HIV), which makes individuals susceptible to *Listeria* infections (Pinner and Broome 1992; Armstrong, 1995; Slautsker and Schuchet, 1999).

Food-borne transmission of *Listeria* cases is 85-95% and this estimate has been widely accepted (Schuchat *et al*., 1992). The robust nature of *Listeria* species coupled with their ability to withstand several food processing treatments, such as pH, temperature and salt conditions, make them frequently end up in the foods consumed by humans (Liu, 2008). The humans are subsequently infected with the *Listeria*. Once inside the human digestive tract, some of the pathogenic *Listeria* (*L. monocytogenes*) overcomes the acidic environment of the stomach, moves across the intestine and subsequently enters the blood circulation, wherein, it migrates to other parts of the body (eg., liver, spleen, brain, and fetoplacental tissue), where it grows and stimulates a range of host responses and clinical diseases (Liu, 2008). Transmission routes of *Listeria* species most especially *L. monocytogenes* via contaminated food leading to infection by the organism is shown in Figure 2.1.

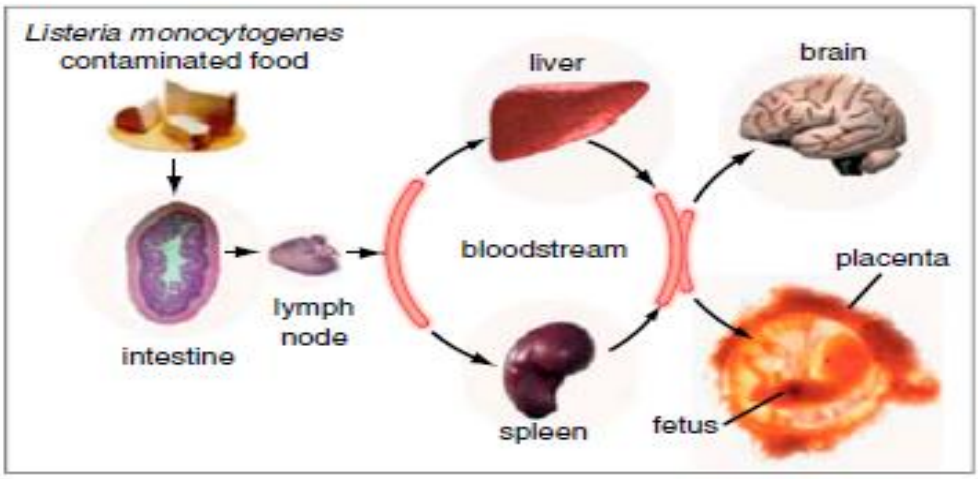

Figure 2.1: The infection by *L. monocytogenes i*n vivo: bacteria, via contaminated food product

Source: Cossart (2011)

This *L. monocytogenes* infection poses serious threat to vulnerable individuals such as pregnant women, neonates, the elderly and immunocompromised individuals (Rocourt and Cossart, 1997), and is among the most virulent foodborne pathogens, with up to 20% of clinical infections resulting in death (Marley, 2013). Members of this group are considered the main consumers of dairy products (Jamali *et al.*, 2013). Listeriosis is usually characterized by gastroenteritis, meningoencephalitis, still birth, septicemia and abortion, premature birth and spontaneous abortions (Tano-Debrah *et al*., 2011).

The infective dose of *Listeria* to cause disease is quite unknown. Large number of *Listeria* bacteria is supposed to be ingested to cause illness in healthy individuals. The estimated numbers stand at 10-100 million viable *Listeria* bacteria in healthy individuals, and 0.1- 10 million CFU in people at high risk of infection (Robert, 2001). The incubation period varies to a significant degree among individuals. Some reports points to 11 to 70 days of ingestion, with a mean of 31 days (Frank, 1999) while others reported 19 and 23 days among pregnant women (Bennett, 2000).

2. 7 Mechanism of survival under negative environmental conditions and virulence

2.7.1 Stress response of *Listeria* species

The virulence potential of *Listeria* is often linked to its ability to withstand the harsh environmental conditions it encountered both in its natural environment and within the host. The *Listeria* species have the ability to withstand external pH, temperature and salt stresses which enable them to survive in almost all environmental conditions (Stack *et al.*, 2008). Existing data on molecular mechanisms of *Listeria* tolerance of heat, acid, and osmotic stresses, which are invariably encountered by this bacterium during its various stages of infection within the mammalian host have been accumulated (Stack *et al.*, 2008). Rocourt and Cossart (1997) reported of the ability of the most pathogenic *Listeria* (*L. monocytogenes*) to adapt to a wide range of environmental conditions such as refrigeration temperature, acidic foods, and high salt foods and inside the host immune system.

2.7.2 Survival of *Listeria* at low temperatures

Listeria has common features of both psychrotroph and mesophile hence can survive at a wide range of temperatures. The ability of *Listeria* and specifically *L. monocytogenes* to survive and grow over the wide range of temperatures (2-45°C) coupled with refrigeration temperatures (2-4°C) are two of the many factors that makes the control of this food-borne pathogen very difficult (Rocourt and Cassart, 1997). Adaptation of the organism to cold stress environment is one of the fundamental attributes of *Listeria* that is essential for their dissemination (Jemmi and Stephen, 2006). *L. monocytogenes* possesses refrigeration survival characteristics and the consumption of refrigerated opened package of sliced pork brawn and opened package of sliced medwurst has ever led to listeriosis (Loncarevic *et al.*, 1997). Refrigeration is one of the methods used in increasing the shelf life of food (Gandhi and Chikindas, 2007), however the organism has survived the refrigeration temperation (Loncarevic *et al.*, 1997) and if this organism can survive and grow at these low

temperatures, then it is important to understand the mechanisms behind its ability to survive at these temperatures. These will help to provide a very effective means of controlling the pathogen (Gandhi and Chikindas, 2007).

2.7.2.1 Changes in *Listeria* membrane composition

The cell membranes of bacterial cells are usually in a fluid, crystalline state presenting an important physical state of maintaining the fluidity of the membrane for effective enzyme activity. Temperature alterations often lead to changes in lipid composition of the membrane to ensure proper membrane fluidity required for effective transport of solutes through the membrane and enzyme activities (Annous *et al.*, 1997). Annous *et al.* (1997) further reported that the cell membrane of *Listeria* is characterized by odd numbers, iso and anteiso, branched-chain fatty acids. The changes of the cell membrane of *L. monocytogenes* with regards to its fatty acid composition in response to low temperature have been greatly studied. Among the changes in the fatty acid composition is the increase in the relative proportion of $C_{15:0}$ at the expense of $C_{17:0}$, when there is a reduction in temperature below optimum (7 °C) (Gandhi and Chikindas, 2007). Beales (2004) also reported that, there is always an increase in unsaturated fatty acid at low temperatures, which help to improve the fluidity of the membrane. An earlier work conducted by Annous *et al.* (1997) indicated that when the growth temperature was changed from 20°C to 5 °C it led to the shortening of fatty acid (a reduction in C17:0) which subsequently led to a switch from iso to anteiso branching. The carbon- carbon interaction that exist between neighbouring chains of the cell membrane decreases as a result of shortening the fatty acid chain length thus helping to keep the maximum degree of membrane fluidity for growth at low temperature (Beales, 2004).

2.7.2.2 Changes in gene expression and induction of proteins by *Listeria*

Bayles *et al.* (1996) reported that *L. monocytogenes* produces cold shock proteins (Csps) and cold acclimation proteins (Caps) which are synthesized during balanced growth at low

temperatures in response to a temperature downshock. Liu *et al.* (2002) indicated that changes in the microbial gene expression occurred as a result of cold acclimation of pathogen. Liu *et al.* (2002) also indicated that an increased expression of mRNA for chaperone proteases like GroEL, ClpP and ClpB showed that these enzymes may be involved in the degradation of abnormal or damaged polypeptides that arise due to growth at low temperatures.

2.7.2.3 Compatible solutes as cryoprotectants

Cryoprotective agents such as glycine betaine and carnitine are compatible solutes that accumulate on *Listeria* (Bayles and Wilkinson, 2000; Stack *et al.* 2008). The uptake and accumulation of these compatible solutes in cells growing at refrigeration temperatures has been investigated by Angelidis and Smith (2003). They reported that, cryoprotection involving solute-mediation encourages the growth of cells subjected under cold stress. Wemekamp-Kamphuis *et al.* (2004) indicated that removing these osmolyte transporters decreased the growth of *Listeria* at low temperature.

2.7.2.4 Role of general stress sigma factor (σB)

The changes in the transcription of genes by the association of alternative sigma factors with the core RNA polymerase has made bacteria to survive under adverse environmental conditions. Gram-positive bacteria such as *Listeria* have been found to possess these alternative sigma factors (Gandhi and Chikindas, 2007; Becker *et al.*, 2000). Becker *et al.* (2000) indicated that the stress sigma factor is stimulated when there is temperature downshift and the *sigB* mutant is unable to accumulated solutes in particular betaine and carnitine in *L. monocytogenes*. The accumulation of cryoprotectants is a major function of sigma B during growth of *L .monocytogenes* at low temperature. The rate of survival and growth of *Listeria* at low temperatures shows the versatility of this emerging pathogen to adapt to varied range of environmental conditions. One great concern about this pathogen is

the ability of it to survive and grow in refrigerated foods, which are eaten without any further processing, particularly soft cheese (Gandhi and Chikindas, 2007).

2.7.3 Survival of *Listeria* under acid stress

Cotter and Hill (2003) indicated that *L. monocytogenes* usually encounter several low pH conditions such as in acidic foods, in the phagosome of the macrophage and during gastric passage. It utilizes several stress adaptation mechanisms by responding to and survives in the low pH environments. *Listeria* species survive and grow in soft cheeses with a pH of 5.5 or above (Rogga *et al.*, 2005). When *Listeria* is exposed to weak acidic pH of 5.5, acid tolerance response is induced, wherein the *Listeria* become resistant to severe acidic environments (O'Driscoll *et al.,* 1999; Gahan *et al.,* 1996) and this phenomenon is termed acid tolerance response (Gahan *et al.*, 1996).

2.7.3.1 Induction of proteins

The protein GroEL synthesized in the course of the growth of *Listeria* at low temperature is also induced under acid stress (Phan-Thanh and Mahouin, 1999). Phan-Thanh and Mahouin (1999) also reported that ATP synthase and various transcriptional regulators are additional proteins induced. Phan-Thanh and Mahouin (1999) again reported that acid-adapted *L. monocytogenes* (pH 5.2, 2h) has increased resistance to alcohol stress, heat shock (52°C) and osmotic shock (25-30%NaCl), which suggest that acid adaptation also provides cross-protection against other stress factors. The cross-resistance of the acid adapted cells to other stresses has essential implications for the food industry, especially since foods commonly encounter sublethal acidic treatments during processing (Van Schaik *et al.,* 1999).

2.7.3.2 Glutamate decarboxylase system

The glutamate decarboxylase (GAD) system is utilized by *L. monocytogenes* to survive under acid stress conditions (Gandhi and Chikindas, 2007). The constituents of the GAD system are

gadA, *gadB* and *gadC* genes (Gandhi and Chikindas, 2007). Cotter *et al.* (2001) indicated that two glutamate decarboxylase have been encoded by *gadA* and *gadB* genes and the *gadC* genes codes for a glutamate/ϒ-aminobutyrate antiporter. Small and Waterman (1998) proposed that decarxylation of glutamate in the cytoplasm of cells leads to a loss of intracellular proton. The loss of the proton from the cell leads to an increase in the pH of the cytoplasm and the release of alkaline ϒ-aminobutyrate into the environment increases the external pH slightly. Cotter *et al.* (2001) reported in their study that, the addition of glutamate to gastric fluid increased the survival of the wild *L. monocytogenes* strain which is of concern in foods containing glutamate. But interestingly, deletion of *gadA*, *gadB* and *gadC* genes led to an outcome of enhanced sensitivity of the strain to low pH. The *gadAB* mutant survival rate in the gastric fluid was reduced. The acid resistance of *L. monocytogenes* and the ability of *L. monocytogenes* to successfully pass through the gastric environment and infect the small intestine are possible due to the functional GAD system (Cotter *et* al., 2001).

2.7.3.3 Role of general stress sigma factor (σB)

Wiedmann *et al.* (1998) showed that *L. monocytogenes* survival rate following exposure to an acidic condition depends on the expression of σB-dependent proteins. Ferreira *et al.* (2003) indicated that the increased resistance and survival of log-phase *L. monocytogenes* to gastric fluid upon subjection to mild acidic conditions were partially dependent on σB. Another studies conducted by Kazmierczak *et al.* (2003) reported that the stress responsive factor σB regulates genes responsible for the survival of *L. monocytogenes* under acid stress conditions and virulence gene expression in this food-borne pathogen.

2.7.4 Survival under osmotic stress

Listeria has the ability to sense and respond appropriately to constantly changing environment, and this is very crucial for its survival and initiation of infection (Sleator and Hill, 2001). Generally, the reaction of microorganisms to osmotic stress concerns both

physiological changes and variations of gene expression patterns and is called osmoadaptation (Hill *et al.,* 2002). One of the common methods used by the food industry for food preservation is the used of salt to lower the water activity; but, the ability of *Listeria* to adapt and survive in high concentrations of salt makes it difficult to control the pathogen in foods (Gandhi and Chikindas, 2007).

2.7.4.1 Induction of proteins

One of the methods used by *Listeria* to tolerate salt stress is a change in its gene expression resulting in an increased or decreased synthesis of various proteins. The 2-D gel electrophoresis was used to study the expression pattern of proteins after inducing salt stress in *L. monocytogenes* and about twelve proteins were identified (Duche *et al.,* 2002). Identical to the two groups of proteins induced in reacting to cold shock conditions (Csp and Cap) (Bayles *et al.,* 1996; Duche *et al*., 2002) reported that salt shock proteins (Ssp) and the stress acclimation proteins (Sap) are rapidly induced by *Listeria* in response to osmotic stress conditions and continue to be over expressed several hours after normal conditions returns. DnaK and Ctc were the two general proteins identified among the Ssps induced in *L. monocytogenes*. DnaK functions as a heat shock proteins thereby stabilizing cellular proteins. GbuA, one of the saps identified, which functions as an osmoprotectant transporter for glycine betaine was induced in response to salt stress (Bayles *et al.,* 1996; Duche *et al*., 2002). Gardan *et al.* (2003) indicated that the Ctc gene is concerned with the resistance of *L. monocytogenes* to high osmolarity in the absence of osmoprotectants such as glycine betaine and carnitine in the medium.

2.7.4.2 Compatible solutes as osmoprotectants

They are highly soluble compounds that do not have net charge at physiological pH and can be accumulated at high concentrations within a cell without affecting cellular functions (Gandhi and Chikindas, 2007). Bayles and Wilkinson (2000) indicated that compounds such

as glycine betaine, proline betaine, acetyl carnitine, carnitine ϒ-butyrobetaine and 3-dimethysulphoniopionate function as osmoprotectants in *L. monocytogenes*. The presences of these compounds have resulted in an up to 2.6-fold increase in growth rate of salt-stressed *L. monocytogenes* as compared to stressed *L. monocytogenes* without any osmoprotectants. Osmolytes from the external environment are taken up by cells as a response to osmotic stress, which help to regain osmotic balance within cells (Bayles and Wilkinson, 2000).

2.7.4.3 Role of general stress sigma factor (σB)

Becker *et al.* (1998) reported that the general stress sigma factor σB in *L. monocytogenes* is vital for the utilization of certain osmoprotectants such as carnitine and betaines. Studies conducted by Gardan *et al.* (2003) showed that the expression of Ctc genes contributing to the osmotic stress response is dependent on σB in *L. monocytogenes*. Moorhead and Dykes (2003) indicated that the sigma factor is an important part of the general stress response of *L. monocytogenes* to adverse environmental conditions, however the extent to which an organism depends on σB for its stress response varies between serotypes.

2.8 Milk production and consumption

The quantity of cows' milk produced and processed in the Northern Region of Ghana is unknown. However, the quantity of the cows' milk produced have been reported by Omore *et al.* (2003) as 10 litres in the dry season and 12 litres in the wet season in Kumasi, and 20 litres in the dry season and 24 litres in the wet season in Accra. Also the actual quantity of cows' milk and milk products consumed in the Northern region is unknown, but there is high availability of raw milk and milk products to domestic consumers largely during the rainy season. In some parts of the Region, there is no milking during the dry season. Statistically, the data on the consumption of milk and milk products can be obtained through production statistics and availability to consumers. In Ghana and other parts of West Africa, raw milk is routinely consumed by the Fulani tribesmen who have easy access to it or process into

various fermented and non-fermented products (Obodai and Dodd, 2006; Akabanda *et al*., 2010, 2013, 2014). A study by Jayarao *et al.* (2006) reported that 42% of dairy producers in US consumed raw milk. It is important to notify that these are not dairy producers. There exists no record of any form of milk from other animals in Northern Region apart from cows' milk. Milk sold to the public is usually boiled or pasteurized by the producers and retailers. The cow's milk is often processed into milk products such as soft cheese, ice cream, naturally fermented milk (*nunu* and *nyarmie*), boiled milk and yoghurt (Omore *et al*., 2003).

2.9 Milk and Milk products handling in Ghana

In Ghana, milk and milk products are usually handled by producer, wholesalers, retailers, hawkers and processors. The vessels used for handling milk in Ghana include plastic buckets/basins, plastic gallons, jerry cans, milk cans/churns, Aluminium basins/bowls, calabashes. The use of plastic containers in handling milk predisposes the milk to bacteria contamination (Omore *et al.*, 2003). Donkor *et al.* (2007) reported that, there is poor hygienic handling of milk in Ghana.

2.10 Milk and Milk product marketing in Ghana

The marketing of milk in Ghana is characterized by several factors. The various milk and dairy product markets differs markedly especially, regarding the types of products handled, the number of intermediaries involved, and the role each plays. The number of intermediaries generally determines the quality of the milk product. The more the intermediaries, the higher the value of the milk product. Each of the intermediaries adds some degree of transformation to the product. In Ghana, more product differentiation exists and there are now markets for local cheese and milk (Omore *et al*., 2004). Much of the milk produced is sold at the farm gate (referred to as *kraals* in Ghana) to consumers, milk vendors, assemblers, processors or wholesalers (Omore *et al*., 2003). The main types of milk agents found in Ghana are illustrated in Table 2.3

Table 2. 3 Main types of Market agents in Ghana

Type of seller	Description
Producer-seller	Producers who also sell their milk. In Ghana, herdsmen or their wives who sell their own milk at the Kraal or in the village, rural town or road-side.
Private Wholesalers/ Assemblers	These buy milk in bulk from producers or from rural Assemblers and sell it to Retailers. No chilling is used. They are bulkers in the marketing chain
Retailers	Retailers present milk to consumers in the smallest desirable quantity, and in a convenient form and location. These are largely open-air road-side sellers
Hawker/Vendors	The vendors are also referred to as traders who collect milk from producers or collectors and sell directly to consumers and other market agents. They may also be the house wives of producers
Fura sellers	These food-drink sellers are mainly in urban centers of Ghana. They buy milk from the kraal, Assemblers or from wholesalers and retail it combined with balls of cooked cereal, Fura, as a snack or meal.
Processors	These buy milk to process in to other products such as yoghurt, ice cream, soft cheese (*wagashi*) and hard cheese. In Ghana, this includes Home processors of *wagashi*, generally wives of stockmen/producers.

Source: Omore *et al.* (2003)

There are several channels for marketing milk in Ghana. It is common to see producers sending their milk to sale point when consumers failed to buy all the milk at the farm gate. Sometimes, the milk is delivered mainly to local processors, assemblers or consumers who have a contract with the milk seller. It is also common to see producers and hawkers carrying milk from house to house or street to street to retail milk in small quantities to consumers. The *woagashie* processors who are not able to process all their fresh milk sometimes give the remaining milk to hawkers, usually their children. Some individuals prefer consuming fresh milk mix with *fura* or *lekri*. As a result the *fura* sellers usually carry fresh milk along with them to use it to prepare the *fura* for people to drink, or they sit at a point with the milk and *fura* to prepare and sell to people to drink. Small quantity of milk is been sold through this channel. Again, a situation where the *Kraals* are far away from town, producers transport milk to their customers in town. Producers/Stockmen, who do not have customers or hawkers

to retail for them usually, choose a place in the centre of the town where milk sellers assemble to sell their milk. Such places have been developed at Kintampo, Kumasi, Nima and Ashiaman (Omore *et al.*, 2003). Figure 2.4 shows the marketing channels of milk in Ghana.

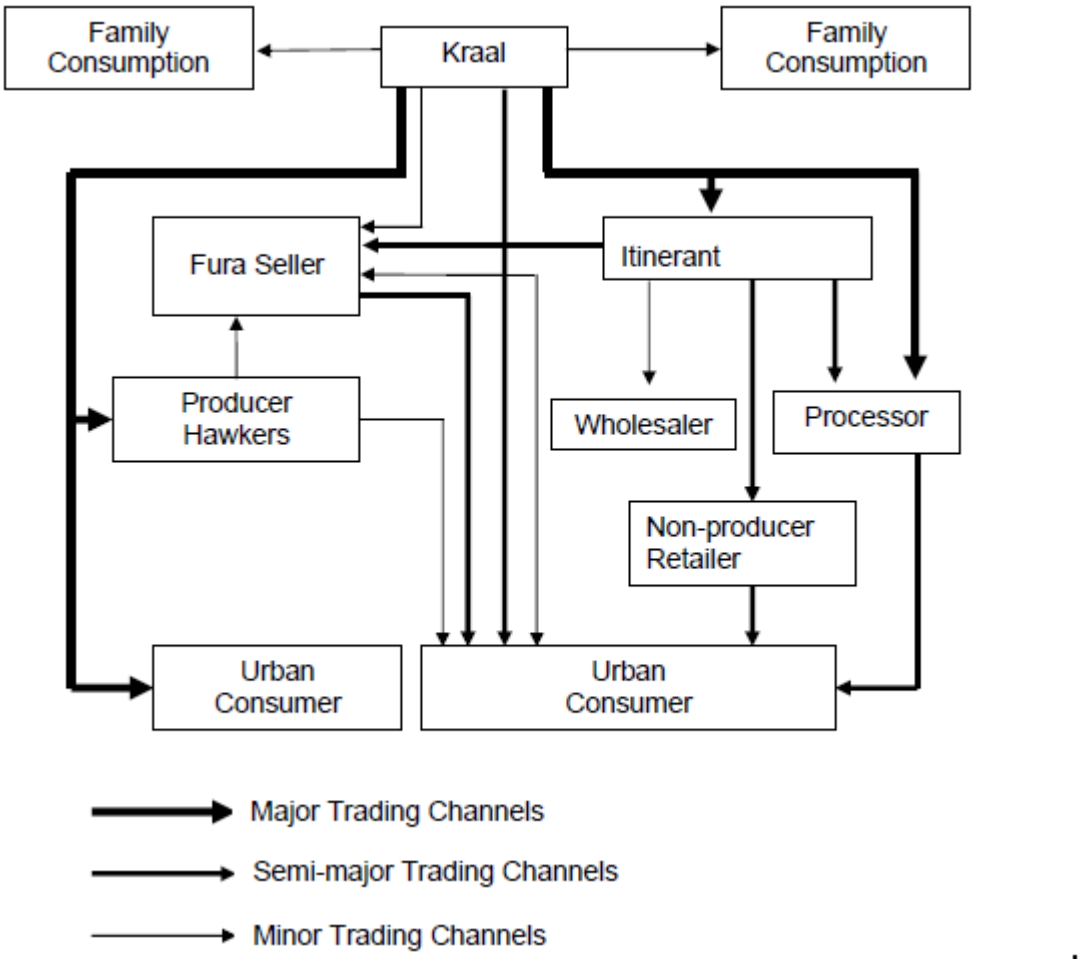

Figure 2.2: Marketing channels of milk in Ghana

Source: Omore *et al.* (2003)

2.11 Rules and Regulations of *Listeria* in ready-to-eat foods

The United States Food and Drug Administration (USFDA) and the microbiological specification for food have adopted a 'zero tolerance' of *L. monocytogenes* in 25g of food sample (Hitchins, 1998; Roberts and Greenwood, 2003). Although in 2008 there was a proposal to loosen up these controls by US Food and Drug Administration (FDA) to allow a

maximum limit of 100/g in frozen and refrigerated RTE foods that do not support the growth of *Listeria* (Lawley, 2013). The European Union regulations allow a maximum limit of 100/g at the end of the shelf life of RTE foods, except those intended for infants and for special medical purposes (Lawley, 2013). Apart from the zero tolerance, contaminating *Listeria* on the food surface to get stressed as a result of routine exposure to conditions that injure and may even kill the bacteria on the surface (Gorski, 2008). The FDA protocol (Hitchins, 2001) and Roberts *et al*. (1995) therefore recommended that every homogenized food sample should be buffered before enrichment.

2.12 Isolation of *Listeria*

The media for isolation of *Listeria* species are selective and differential. The media could be in broth or solid form (Chukwu, 2007).

2.12.1 Nonselective media

Listeria species have been detected directly from nonselective enrichment broths using PCR. The incorporation of the pre-enrichment of food samples by non-selective media is necessary for the recovery of stressed organisms and detection of low numbers in food. The pre-enrichment achieves the sensitivity of allowing one *Listeria* organism to grow to a detectable level of approximately $10^4 – 10^5$ CFU ml-1 (Gasanov *et al*., 2005; Law *et al*., 2015a). Hudson *et al*. (2001) indicated that isolation methods that do not use enrichment require more cells to be present before detection can be achieved. Enrichment provides enough DNA for PCR detection. Some pre-enrichment and enrichment media include half Fraser, buffered peptone water, triptic soy yeast extract, universal pre-enrichment broth and Fraser broth. Many of these pre-enrichment and enrichment media are nonselective. The enrichment steps adopted by the food microbiologist do not give rise to *Listeria* in the food sample being affected by the natural flora of the contaminated food (Bess *et al.,* 2005). This is because they contain

inhibitory substances (Gorski, 2008). The pre-enrichment and enrichment steps allow the resuscitation of stressed organisms before plating on selective agar. Many researchers have tried to compare the various non-selective broths for the isolation of *Listeria* and *L. monocytogenes.* Jersek *et al.* (2005) conducted a comparative evaluation of different enrichment media for the isolation of bacteria of the genus *Listeria* and indicated that buffered peptone water and yeast extract broth failed to allow detection of *L. monocytogenes* after 48 h of incubation when PCR was done directly from their enriched broths, but was detected in universal pre-enrichment broth and half Fraser broth. They (Buffered peptone water and yeast extract broth) however allowed detection from suspected *Listeria* colonies on palcam (selective) agar plates. Hudson *et al.* (2001) detected the listeriolysin O gene of *L. monocytogenes* in 25g of ham sample after 24 h incubation of the ham in brain heart infusion broth using a combined immunomagnetic separation (IMS)/polymerase chain reaction (PCR).

2.12.2 Selective agar media

A number of selective media have been introduced for microbiological analysis of food samples after they have been enriched in nonselective broths. The selective agars relied on a combination of selective agents like phenylethanol, antibiotics, lithium chloride and glycine anhydride (Adams and Moss, 2008). It is however important to note that a number of *Listeria* selective agars are formulated from McBride *Listeria* agar. They include center for disease control (CDC) modified McBride *Listeria* agar (MLA), Acriflavine-Ceftazidimine agar (ACA), FDA modified McBride agar (MMA), Lithium chloride-phenylethanol moxalactam (LPM) agar (McBride and Girard, 1960; Lee and McClain, 1986). Two differentials *Listeria* media (oxford agar and palcam agar) have been developed by Curtis *et al*. (1989) and Van-Netten *et al*. (1988) for presumptive identification of *Listeria* species. Many researchers have tried to compare the various selective solid agar media for the isolation of *Listeria* and *L. monocytogenes.* Jamali *et al.* (2012) conducted comparative studies on the efficiencies of

three selective cultural media in the detection of *L. monocytogenes* and detected that the efficiencies of detection on chromogenic agar, *Listeria* selective agar (oxford agar) and palcam agar after 48 h of incubation were 98.8, 93.2 and 90.0 respectively. The observations inferred from the above and other literatures show that there has been enhancement of both the nonselective enrichment and selective media thereby enabling them to detect low numbers of *Listeria* in samples, to shorten culture time period and detection of stressed or heat injured organisms. The cultural method is the classical approach in the identification and isolation of bacteria. In this approach several steps are involved which are designed to isolate and identify the *Listeria* species at the genus and species levels respectively (Chukwu, 2007).

2.12.2.1 Culturing *Listeria* on PALCAM agar and OXFORD agar

After forty eight hours of incubating food samples on palcam agar, the colonies formed would show grey green with a black sunken centre with a black halo against a cherry background (Oxoid, 2006; Mcluachlin and Rees, 2009). Similarly, on the oxford agar, colonies formed would appear to be surrounded by black halos (Oxoid, 2006; Mcluachlin and Rees, 2009). The grey green colonies with black halos formed on the palcam agar plates and grey-green surrounded by black halos on the oxford agar are presumptive evidence of *Listeria* species (Gorski, 2008). The enzyme esculinase β-D glucosidase produced by all *Listeria* species hydrolyzes aesculin, resulting in the formation of 6, 7 – dihydroxycoumarin and forms a complex with the ferric ammonium citrate which is a component of the media (Gorski, 2008). This compound (ferric ammonium citrate) causes the blackening of the media (Gorski, 2008).

The nutrients compositions of both media (palcam and oxford) are almost the same, though slight differences exist between them which can subsequently affect their performance. Both media (palcam and oxford) have a battery of inhibitory substances most of which are antimicrobials. The presence of these inhibitory substances increases the detectability of the

Listeria. Acriflavine inhibits the growth of other gram positive bacteria especially gram positive cocci while cycloheximide inhibits the growth of fungi (Gorski, 2008). On the other hand, Nalidixic acid inhibits the growth of gram negative bacteria (Gorski, 2008). It is important to note that the cycloheximide and acriflavine are used in both the enrichment and the plating media. The presence of Lithium Chloride (LiCl) inhibits the growth of enterococci and other gram negative bacteria (McBride and Girard, 1960; Gorski, 2008). Colistin and polymixine B rupture the cell membrane of gram negative bacteria (Gorski, 2008). Ceftazidime (present in palcam agar) and cefotetan (present in oxford agar) are other antibiotics active against many Gram-negative bacteria (Gorski, 2008). Fosfomycin in oxford agar is an organic phosphonate that interferes with Gram-negative bacterial cell wall synthesis thereby inhibiting their growth (Gorski, 2008).

Thereafter, the colonies are subjected to confirmation by biochemical testing and characterization and further by PCR analysis. The two plating media does not distinguish between *L. monocytogenes* from other *Listeria* species which are been noted to be non-pathogenic (El Marrakchi *et al*., 2005).

2.12.3 Biochemical methods for confirmation of *Listeria* species

The confirmation of positive *Listeria* isolates by biochemical methods is important for identification and subsequent speciation of the genus. Several biochemical methods have been accepted internationally (Chukwu, 2007), but the most importan tests are assessments of hemolytic activity and carbohydrate fermentation patterns (Gorski, 2008). Others are Gram reaction, tumbling motility test, catalase production, oxidase test, CAMP (Christie, Atkins, Munch–Petersen) test and nitrate reduction (Roberts and Greenwood, 2003). Detailed description of the procedures for the biochemical reactions are described below.

2.12.3.1 Gram's stain

This test put all bacteria in to two groups based on the ability of a particular bacterium to retain the first stain used in the procedure when a decolorizing agent such as ethanol or acetone is added (Roberts and Greenwood, 2003). The following reagents and their chemical combinations are used in carrying out the staining: crystal violet (1% aqueous solution), lugols iodine (1% iodine, 2% potassium iodide), acetone/alcohol mixture: 20% acetone/80% methylated spirit and safranin solution (0.5% aqueous solution) (Roberts and Greenwood, 2003). The test procedure for the gram staining is given below.

- ✓ A single pure colony of the bacteria is suspended in sterile distilled water on a clean. microscope slide using sterile loop.
- ✓ The bacterium is first air dry on the slide and fixed using flame.
- ✓ The fixed bacterium is allowed to cool.
- ✓ The fixed bacterium is flooded with crystal violet solution for 30 seconds.
- ✓ The stain is washed off using water.
- ✓ The slide is flooded with lugols iodine solution for 30 seconds.
- ✓ The lugols iodine solution is washed off using water.
- ✓ The fixed bacterium is decolorized with acetone/alcohol for few seconds.
- ✓ The acetone/alcohol is washed off using water.
- ✓ Again, the fixed bacteria is Flooded with safranin solution for 1 minute.
- ✓ The safranin solution is washed off using water.
- ✓ The bacterium on the slide is air dried.
- ✓ A drop of immersion oil on the fixed bacteria is added.
- ✓ The slide is then examined under the microscope using the oil immersion objective lens (×100)
- ✓ The bacteria that appear dark purple are Gram positive; those that appeared as pink

are Gram negative.

Source: Roberts and Greenwood (2003)

2.12.3.2 KOH solubility test

The KOH reaction put all bacteria into two broad groups which include gram positive bacteria and gram negative bacteria. The groupings depend on the ability of a cell to produce a mucous strand. Gram negative bacteria cell wall is easily disrupted when exposed to dilute alkali solutions (Davis, *et al.,* 1968). The disruption of the cell wall in the KOH solution makes the solution viscous due to the release of relatively unfragmented threads of deoxyribonucleic acid. As the loop is raised a string of mixture would follow. The stringing of the mixture of the bacteria and KOH within the first 30 s of mixing indicates the bacterium is gram negative. Other bacteria do not display any reaction (absence of stringing) when suspended in KOH solution (Halebian *et al*., 1981). These bacteria are classified as gram positive bacteria. This method stand as an accurate alternative test for Gram's staining (Halebian *et al*., 1981). The test procedure for the KOH solubility test is given below.

- ✓ Two drops of a 3% solution of potassium hydroxide is placed on a glass slide preferably slides with concave wells.
- ✓ Two-mm loopful of bacterial growth obtained from a 48 h culture is added.
- ✓ The bacterial growth and the KOH solution are stirred in a circular motion to ensure they are well mixed.
- ✓ The mixture is touched with a loop and the loop raised occasionally from the surface of the slide.
- ✓ Stringed formation is an indicative of gram negative bacteria while the absence of stringing is an indication of gram positive.

Source: Halebian *et al*. (1981)

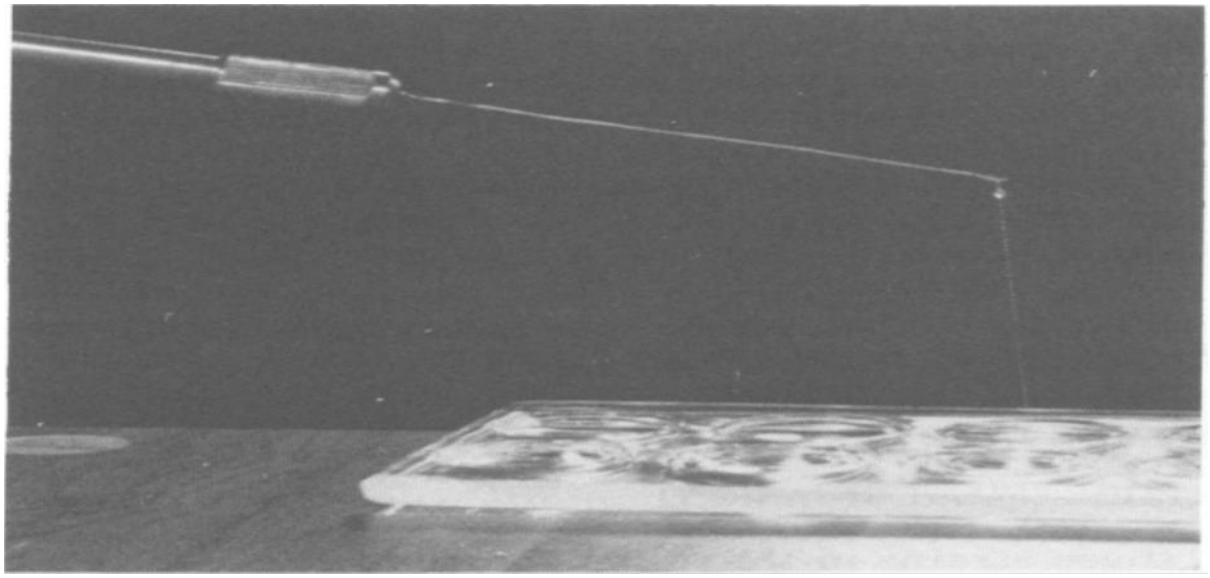

Figure 2.3: A positive KOH reaction characteristic of gram-negative bacteria.

Source: Halebian *et al*. (1981)

2.12. 3.3 Beta-haemolysis reaction test

When suspected bacteria such as *Listeria* are growing on blood agar media, they produce haemolysins which diffuse into the medium and affect the red blood cells. The effect may be β-haemolysis with a characteristic of green zone with blood cells still intact or as beta-haemolysis with a clear colourless zone where the cells are completely lysed (Barrow and Feltham, 1993). The test procedure for beta-haemolysis is given below.

- ✓ A blood agar plate is inoculated with the test organism.
- ✓ The plate is incubated for 18-24 h at 37°C.
- ✓ The plate is examined for visible zones of haemolysis around the test organism.

Source: Roberts and Greenwood (2003)

2.12.3.4 Catalase test

Respiration as a life process is undertaken by all living organisms. *Listeria* as an aerobe and facultative anaerobe bacteria use oxygen for respiration. In the course of respiration by *Listeria,* hydrogen peroxide is produced as a metabolic waste product. The hydrogen

peroxide produced is quickly broken down into water and oxygen as it is toxic to the cells and hence can be suicidal. The breakdown of the hydrogen peroxide into water and oxygen is catalyst by the enzyme 'Catalase' (Barrow and Feltham, 1993). A colony that produces effervescence as the oxygen is released scored such a colony as a catalase positive (Roberts and Greenwood, 2003). The test procedure for catalase test is given below.

- ✓ A drop of 3% hydrogen peroxide solution is placed on a glass microscope slide.
- ✓ A bacteriological loopful of 24-48 h of the test organism is added to the hydrogen peroxide.
- ✓ It is observed for the production of gas bubbles.
- ✓ A test organism that produces gas bubbles is an indication of catalase positive bacteria while absence of gas production in any test sample is an indication of catalase negative bacteria.

Source: Roberts and Greenwood (2003); Chukwu (2007)

2.12.3.5 Motility test

Cells showing the characteristics of *Listeria* after been confirmed by Gram's stain as gram positive and catalase reaction as catalase positive are further subjected to motility in motility agar (Chukwu, 2007). The test procedure for motility is given below.

- ✓ Nutrient broth is prepared in a small tube.
- ✓ The nutrient broth is inoculated by the test organism.
- ✓ This is incubated at appropriate temperature. For *Listeria* species the incubation is done at 21°C for 4–6 h.
- ✓ A drop of the broth is placed on the surface of a microscopic glass slide and cover with a glass cover slip.
- ✓ Slide is examined by optical microscopy for motility of the test organism.

- ✓ *Listeria* species show a typical 'tumbling' / umbrella motility at 21°C but not at 37°C.

Source: Roberts and Greenwood (2003)

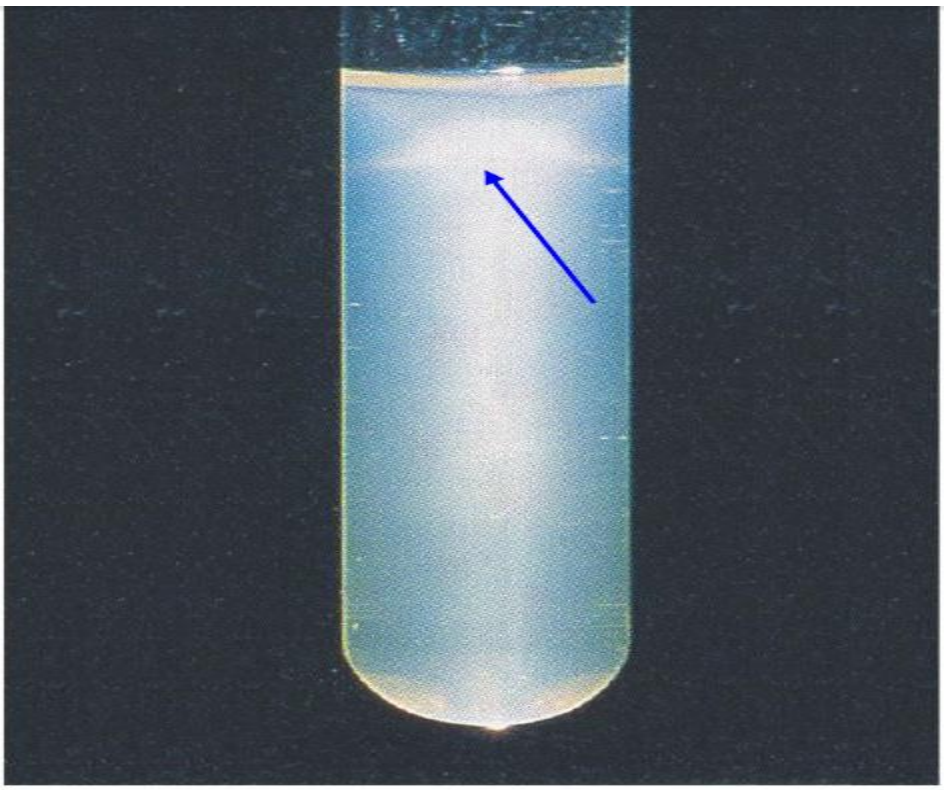

Figure 2.4: A typical umbrella growth characteristic (arrow) in motility medium showing *L. monocytogenes* at room temperature
Source: Chukwu (2007)

2.12.3.6 Acid production from sugars

Test organisms such as *Listeria* are always tested for their ability to ferment Glucose, Rhamnose, Xylose, Lactose, Sucrose and Manntol to produce acid and gas. The test procedure for acid production from sugars is given below

- ✓ Ten percent of the required sugar solution is prepared.
- ✓ The solution is sterilized by autoclaving at 115°C for 10 min.
- ✓ Ten ml of the required sterile 10% sugar solution and 1–2 ml of Andrade's indicator solution is added to 90 ml sterile peptone water.

- ✓ Four–five ml volume is aseptically transferred to sterile bijoux bottles or test tubes incorporating with an inverted Durham tube to check for gas production.
- ✓ This is incubated overnight at 37°C to check for sterility.
- ✓ The bottle or tube of peptone water sugar is inoculated by a pure culture of the test organism.
- ✓ This is incubated at 37°C for up to 24 hours in the case of *Listeria*.
- ✓ The development of a pink coloration indicates the production of acid and the presence/absence of gas in the Durham tube included.

Source: Roberts and Greenwood (2003)

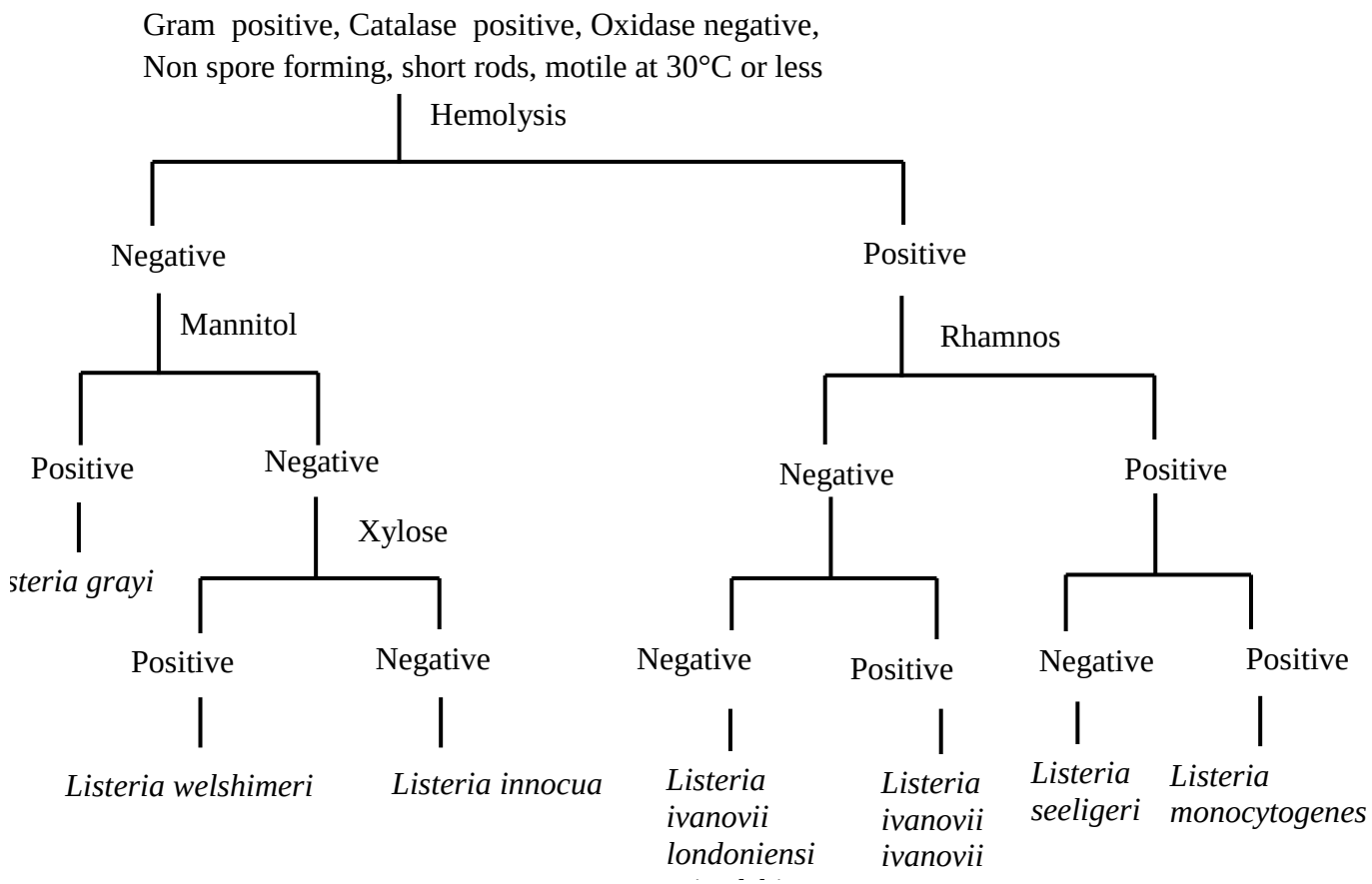

Figure 2.5: Schematic identification of *Listeria* species based on acid production and haemolysis.
Source: Gorski (2008)

2.12.3.7 CAMP (Christie, Atkins, Munch–Petersen) test (*Listeria*)

Haemolytic species of *Listeria* and non-haemolytic ones are differentiated using reactions in the CAMP test. Beta (β) toxin of *Staphylococcus aureus* and an exofactor produced by *Rhodococcus equi* are used in conjuction with synergistic reactions of haemolysis or *Listeria* species. Plates were usually incubated at 37°C for 24 h (Chukwu, 2007). The detailed test procedure is given below.

- ✓ Two plates of about 10 ml nutrient agar with a thin layer (3-4 ml) of 5% sheep blood agar are prepared.
- ✓ The recommended standard strain of *S. aureus* (NCTC 1803) is streaked across the centre of one of the plates and the recommended standard strain of *R. equi* (NCTC 1621) is streaked across the centre of the other plate.
- ✓ The test organism is inoculated on each plate by streaking at right angles to within 1–2 mm of the standard organisms.
- ✓ The plates are incubated at 37°C for 24 h.
- ✓ They are examined for zones of haemolysis of the test organism by either, both or neither of the standard strains where the two cultures are closest together.
- ✓ There is an enhancement of haemolysis when *L. monocytogenes* is grown next to *S. aureus* than when grown adjacent to *R. equi*.

Source: Roberts and Greenwood (2003)

2.13 Virulence factors of *L. monocytogenes*

2.13.1 Internalin A (*InlA*)

This is an acidic surface protein of 800-amino acid required for the internalization of *L. monocytogenes* into the host epithelial cells, macrophages, fibroblasts and epithelial cells

(Parihar *et al*., 2004). The E-cadherin present on the surfaces of hepatocytes, dendritic cells, brain microvascular endothelial cells, and the epithelial cells lining the choroid plexus and placental chorionic villi, easily makes these regions targerted for *Listeria* infection (Va´Zquez-Boland *et al.,* 2001). The E-cadherin present on the host cell membrane is the main receptor for this protein (*InlA*). E-cadherin is species-specific due to its amino acid (proline) at location 16 and is a calcium dependent cell adhesion glycoprotein (Parihar *et al*., 2004).

2.13.2 Listeriolysin O (LLO)

Listeriolysin O is the major determinant of listerial virulence and it is responsible for the hemolytic activity of *L. monocytogenes* (Kuhn *et al*., 2008). LLO is a 58-kDa protein that belongs to the family thiol-activated, cholesterol-dependent, pore-forming toxins (CDTX) for which streptolysin O is the prototype (Kuhn *et al*., 2008). The LLO received its name from the CDTX, and has a narrow pH range (4.5 to 6.5) (Geoffroy *et al.,* 1987). *Listeria* engulfed by the host cell is usually encased within an intracellular vacuole bounded by a membrane (Parihar *et al*., 2004). The pore-forming toxin gives *L. monocytogenes* the ability of lysing the vacuolar membrane in the host cell thus enabling its escape from the vacuole (Parihar *et al*., 2004). The host cellular responses such as interleukin-1 secretion in macrophages; apoptosis; and cell adhesion protein expression; cytokines in spleen cells and mitogen-activated kinase in HeLa cells lines are stimulated as a result of LLO activation (Parihar *et al*., 2004). The response depends largely on Ca^{2+} (Parihar *et al*., 2004). The listeriolysin O is usually endoded by the *hly*A gene in the *L. monocytogenes* (Mengaud *et al*., 1988)

2.14.4 Molecular detection of *Listeria* species by Polymerase Chain Reaction (PCR)

The safety of food products consumed by humans is very critical. It is important to assess the safety of food products by detecting pathogens and other microbial contaminants in the foods. It is also important to identify and use a quick and reliable method in detecting the pathogens

in foods samples. The traditional method for the detection of pathogens including *Listeria* relies largely on growth in culture media, followed by isolation, biochemical identification, and sometimes serology. The efficiency, specificity and sensitivity of detecting micro-organisms, have recently been improved through technological advances. The polymerase chain reaction (PCR) assay has been employed by detecting technologist for the detection of food-borne pathogens. Polymerase chain reaction (PCR) involves in situ DNA replication process allowing for the exponential amplification of target DNA in the presence of synthetic oligonucleotide primers and thermostable DNA polymerase (Wang *et al*., 2000; Liu *et al.*, 2008). The PCR approach is very elegant and distinct among the diverse approaches used in determining the safety of food. It is widely applied in both research and clinical laboratories because of its versatility and robustness. In its practical form, two short single - stranded oligonucleotide primers (usually 20-30 bases in length) or probes that flank the front and rear ends of specific DNA target are hybridized to the specific sequence or template, which is subsequently enzymatically amplified by the Taq polymerase enzyme using a thermocycler (Barrett *et al*., 1997; Liu *et al.,* 2008). The primers are oriented such that their 3' ends points towards each other. The PCR techniques can amplify a single copy of DNA for about a million-fold in less than 2 hours, thus, the potential of the PCR to eliminate or greatly reduce the need for cultural enrichment (FAO, 2010). The identification of virulence nucleotide sequences for use as molecular markers in pathogen detection has been made possible through genetic characterization of the genome sequence information.

The process of amplification during PCR often begins at a high temperature (eg., 94°C) to convert the double stranded DNA template into single strands, follow by relatively low temperature (eg., 55°C) to enable annealing between the single-stranded primer and the single stranded template, and then at 72°C to enable DNA polymerase extending along template (Figure 2.6). Thus PCR process involves three (3) major stages which include

denaturing, annealing and extension. During the denaturing stage, the DNA is melted to convert the double stranded DNA to single stranded DNA, in the annealing; primers are annealed to the target DNA sequence and the DNA extension involves addition of nucleotide from the primers under the influence of DNA polymerase. The oligonucleotide primers are designed such that they hybridize to regions of DNA flanking a desired target gene sequence of the organism by the aid of the Taq polymerase, usually in the presence of free deoxynucleotide triphosphate, resulting in the duplication of the starting target material. When the product of the DNA duplexes melt and the process is repeated, the result is an exponential increase in the amount of target DNA as shown in Figure 2.7

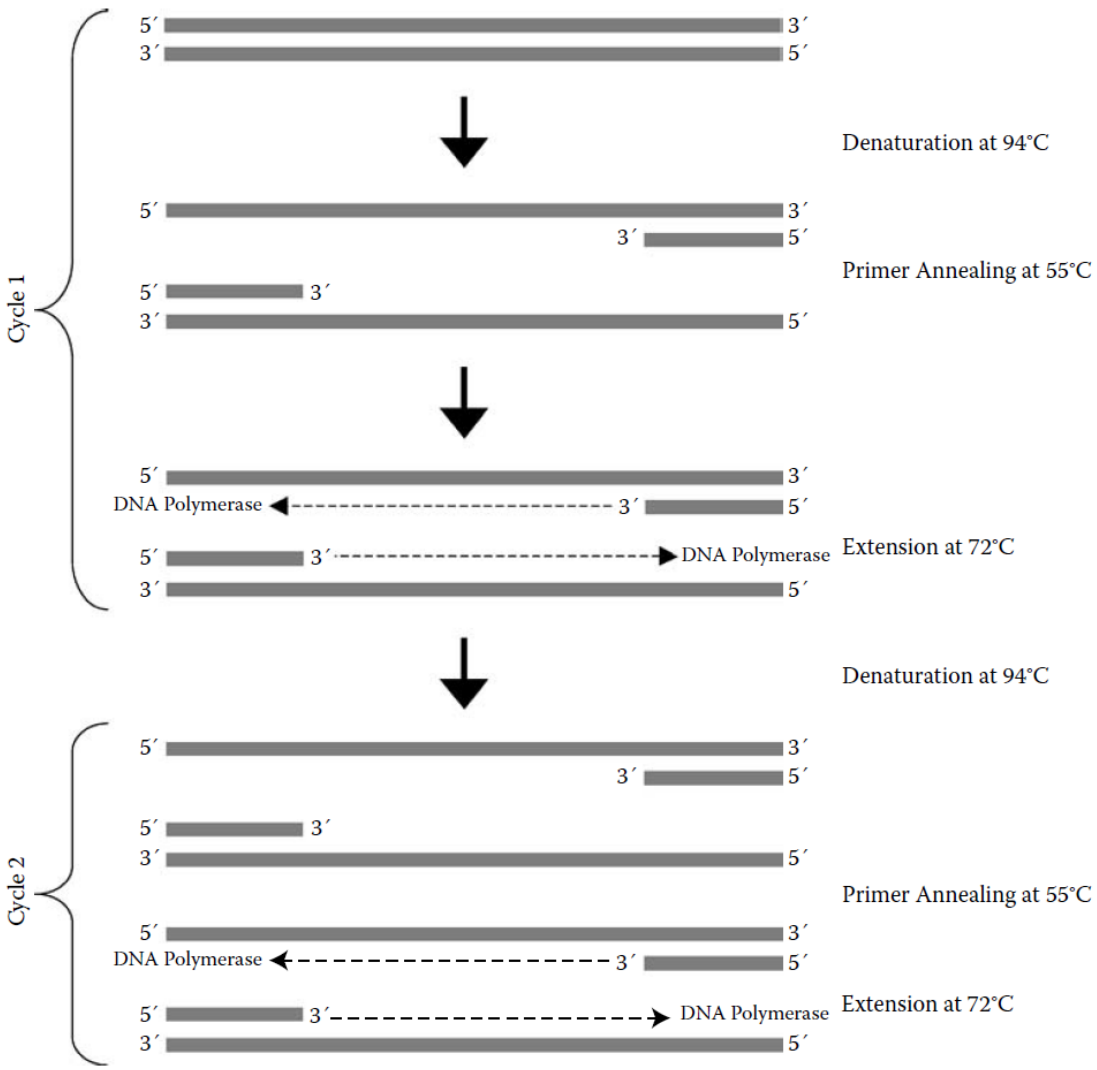

Figure 2.6: Schematic overview of polymerase chain reaction
Source: Liu *et al.* (2008)

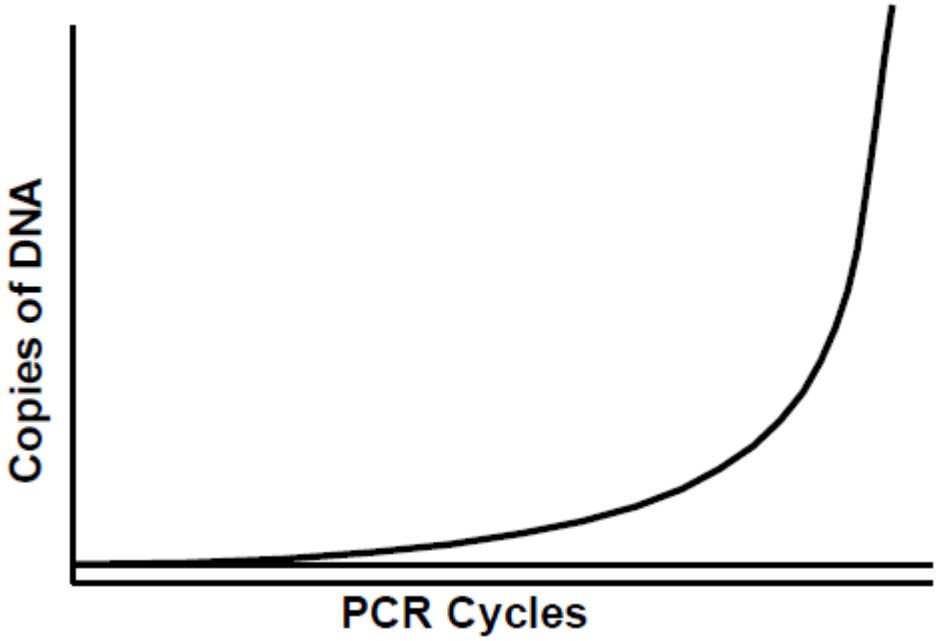

Figure 2.7: Exponential increase in Target DNA
Source: Steffan and Atlas (1991)

The usefulness of PCR is immeasurable and incomparable. PCR demonstrates exquisite specificity, unsurpassed sensitivity, rapid turnover and amenableness to automation for high-throughput testing due to its ability to selectively amplify specific targets present in low concentration (Liu *et al*., 2008). It has the ability to identify organisms in both cultured and non-cultured samples. PCR is therefore noted to be highly sensitive, versatile, and reproducible technique for the identification of *Listeria* species from other bacteria (Liu *et al*., 2008). The polymerase chain reaction assays ensure routine rapid detection, identification and differentiation of food-borne pathogens. Situations where phenotypic characteristics are ambiguous and wrongly interpreted are avoided with the use of PCR. Polymerase chain reactions assays have been extensively used in DNA cloning, diagnosis of hereditary and infectious diseases, identification of genetic fingerprints, and detection and diagnosis of infectious diseases. Another important role of PCR is in its ability to identify typical bacterial strains that exist in viable but nonculturable conccoid form (eg. *Campylobacter* species) which are usually failed to be detected by the conventional method (Magistrado *et al*., 2001).

It is however important to state that, some PCR's may not be suitable for processed and certain foods due to amplification from DNA originating from both viable and nonviable cells (Sails *et al*., 1998; Wang *et al*., 2000). PCR techniques can be inhibited by components of enrichment broth and DNA extraction solution, concentration of the PCR mixtures (primers, DNA templates, dNTP's and Mg_2), temperature and cycling conditions (Rossen *et al*. 1992; Wilson, 1997; Wassenaar and Newell, 2000). Table 2.4 summarizes the advantages and disadvantages of some commonly available molecular techniques for identifying foodborne pathogens.

Table 2.4: Advantages and disadvantages of some commonly molecular techniques involving nucleic-acid base

Detection Method	Advantages	Disadvantages	References
Simple PCR	✓ High sensitivity ✓ High specificity ✓ Automated ✓ Reliable results	✓ Affected by PCR inhibitors ✓ Require DNA purification ✓ Difficult to distinguish between viable and non-viable cells	Mandal *et al.*2011; Zhang, 2013; Park *et al*., 2014
Multiplex PCR	✓ High sensitivity ✓ High specificity ✓ Automated ✓ Reliable results ✓ Detection of multiple pathogens	✓ Affected by PCR inhibitors ✓ Primer design is crucial ✓ Difficult to distinguish between viable and non-viable cells	Mandal *et al.*2011; Zhang, 2013; Park *et al*., 2014
Real-time PCR	✓ High sensitivity ✓ High specificity ✓ Rapid cycling ✓ Reproducible ✓ Does not require post-amplification products processing ✓ Real-time monitoring PCR amplification products	✓ High cost ✓ Difficult for multiplex real-time assay ✓ Affected by PCR inhibitors ✓ Difficult to distinguish between viable and non-viable cells ✓ Requires trained personel ✓ Cross contaminations will occur	Mandal et al.2011; Zhang, 2013; Park *et al*., 2014
Nucleic acid sequence-based amplification	✓ Sensitivity ✓ Specificity ✓ Low cost ✓ Does not require thermal cycling conditions ✓ Able to detect viable microorganisms	✓ Require viable microorganisms ✓ Difficult in handling RNA	Lauri and Mariani, 2009; Zhao *et al.*, 2014
Loop-mediated Isothermal amplification	✓ High Sensitivity ✓ High specificity ✓ Low cost ✓ Easy to operate ✓ Does not require thermal cycling system	✓ Primer design is complicated ✓ Insufficient to detect unknown or unsequence targets	Zhao *et al*., 2014

Source: Law *et al.* (2015b)

2.15 Milk and Milk products as vehicles of listeriosis

Raw milk and dairy products such as pasteurized milk and soft cheeses have been implicated as major outbreaks of listeriosis. This is because they are frequently contaminated with *L. monocytogenes.* The incidence of *L. monocytogenes* in raw milk and dairy products have been reported in many studies (Pintado *et al*., 2005; Abrahao *et al.,* 2008; Arslan and Ozdemir, 2008; Awaisheh, 2009; AL-Shamary, 2010; Tano-Debrah *et al*., 2011; El-Shenawy *et al.,* 2011; Mugampoza *et al*., 2011; Yakubu *et al*., 2012; AL-Ashmawy *et al*., 2014; Meshref *et al*., 2015; Dabash *et al*, 2016) all over the world, including Ghana. Also, listeriosis outbreaks have occurred in some parts of the world of which milk and milk products were associated. Pasteurized milk was implicated in an outbreak of listeriosis in Massachusetts in 1983 involving 42 adult and 7 perinatal cases with an overall mortality rate of 29% (Adams and Moss, 2008). The origin of the milk was from farms where bovine listeriosis is known to have occurred at the time of the outbreak. Perhaps, improper pasteurization at the dairy was a factor that gave rise to the concern that *L. monocytogenes* might display marked heat resistance in some instances (Adams and Moss, 2008). Pasteurized milk has again been implicated with an outbreak of *L. monocytogenes* infection (Gellin *et al.,* 1991; Lyytikainen *et al.,* 2000). Processing raw milk in to a product before pasteurization does not reduce the microbial load and thus poses risk to the consuming population (Morobe *et al*., 2009).

A Mexican-style soft cheese which had been contaminated with raw milk was the vehicle of listeriosis outbreak in California in 1985 (Adams and Moss, 2008). It was reported that 142 cases comprising of 93 perinatal and 49 adults with mortality rate of 34% was recorded. Another outbreak of listeriosis occurred during the period 1983-87 with 122 cases and 31 deaths (Adams and Moss, 2008). This was associated with the Swiss cheese Vacherin Mont d'Or. A very recent outbreak of listeriosis associated with soft cheese consumption occurred

in Sweden where 33 cases were recorded. The reasons so far advanced for the association with soft cheese is due to the cheese ripening process. The ripening process of cheese is characterized by microbial utilization of lactate and release of amines increasing the surface pH allowing *Listeria* to multiply to dangerous levels, and thus causing the listeriosis (Adams and Moss, 2008). De Roin *et al.* (2003) showed that *L. monocytogenes* survived 151 days at 10°C and 73 days at 22°C in soil thus highlighting the importance of dust as a vehicle for recontamination of milk, which when consumed may lead to listeriosis. Table 2.5 showed examples of outbreaks of human listeriosis related to milk and dairy products.

Table 2.5: Reported listeriosis outbreaks in Europe caused by milk or dairy products

Year	Country	Product type	Number of case (deaths)	Serotype	References
1949-1957	Germany	Raw milk	about 100	[a]NA	Seeliger, 1961
1983-1987	Switzerland	Soft cheese[b]	122(33)	4b	Bula *et al.*, 1995
1986	Austria	Raw milk/vegetables	28(5)	1/2a	Allenberger and Guggenbichler, 1989
1989-1990	Denmark	Blue-mold cheese/hard cheese	26 (6)	4b	Jensen *et al.*, 1994
1995	France	Soft cheese[b]	37 (11)	4b	Goulet *et al.*, 1995; Rocourt *et al.*,1997
1997	France	Soft cheese[b]	14	4b	Jacquet *et al*., 1998
1998-1999	Finland	Butter	25 (6)	3a	Lyytikainen *et al*., 2000
2001	Sweden	Soft cheese[b,c]	33	1/2a	Carrique-Mas *et al*., 2003

[a]Data not available
[b]Vehicle of infection identified
[c]Mixed etiology possible

Source: Lunden *et al.* (2004)

2.15.1 Occurrence of listeriosis in humans in Ghana

Even though there have been cases of outbreaks of human listeriosis in different parts of the world in which milk and milk products have been implicated, however, there has never been

any reported outbreak of human listeriosis in Ghana. The first evidence of the occurrence of listeriosis in Ghana was reported to have occurred among herds of sheep on the Animal Research Institute field station at Pokoase on the Accra Plains (Osei-Somuah *et al*., 2000). However, symptoms of *L. monocytogenes* infections have been documented among patients reporting in some health facilities in Ghana. In 2009, more than 80% of patients reporting in the health facilities in the Ashaiman district where raw milk consumption is high were meningitis. Also, in 2007, 73% of cases reported in these same health facilities in the district were meningitis and spontaneous abortions (Tano-Debrah *et al*., 2011). Spontaneous abortion, one of the symptoms suggestive of *L. monocytogenes* infections has again been reported among the raw milk retailer-consumers at Ashaiman municipality (Appiah, 2012).

2.15.2 Occurrence of listeriosis in humans in West Africa

Listeria has been isolated from adult female in Nigeria (Eyo *et al.*, 1969). This was considered as the first case of *Listeria* infection in Nigeria and the West African sub-continent as a whole (Nwaiwu, 2015). Again, neonatal listeriosis was reported when *L. monocytogenes* was isolated from a 2-day old neonate who developed meningitis after contracting the organism from the mother (Nwaiwu, 2015). In all these instances the individuals were successfully treated in the hospitals, with the adult female receiving chloramphenicol and prednisone, and both the neonate and the mother receiving ampicillin treatments respectively (Nwaiwu, 2015). Onyemelukwe *et al.* (1983) also reported 27% mortality rate from 19 patients with clinical symptoms of meningitis and meningoencephalitis. They were tested positive for *L. monocytogenes* in a 1 year prospective study (Nwaiwu, 2015).

2.16 Behaviour of *Listeria* in milk and milk products

Listeria species including *L. monocytogenes* has the ability to grow at refrigeration temperature, though growth is slow. However, growth is accelerated as temperature rises

(King *et al.*, 2014). Gay and Amgar (2005) reported that *L. monocytogenes* growth is more slowly in raw milk and supplemented milk than in pasteurized milk. They indicated that the populations of *L. monocytogenes* increased by 2 to 3.8 log and 0.8 to 2.3 log in pasteurized and raw milk respectively, with both milk been kept at the same conditions. They again reported that *L. monocytogenes* growth in raw milk camembert cheese (RMC) was about twice as slow as in Camembert cheese made from pasteurized milk (PMC). They showed that, average lag phase (Lag) was 15 d in PMC and 34 d in RMC. Also, Dijkstra (1971) reported that 4 (11%) out of 36 naturally contaminated raw milk (obtained from cows that experienced *Listeria*-related abortions) samples stored at 5°C for 9 years contained the pathogen *L. monocytogenes*. There was a decreasing trend in the number of contaminated samples up to the 9^{th} year, with 32, 16 and 4 being free of *L. monocytogenes* after 6 months, 2 years and 8-9 years of storage. Hence, a number of cleaning and sanitizing programs were implemented for all phases of milk production (Ryser, 1999). *L. monocytogenes* takes at least five days for its concentration to be increased by 1 $\log_{10}$ CFU/ml at 4°C (King *et al*., 2014). An increase in temperature beyond 4°C increases the rate of growth of the organism, even within the shortest possible time (King *et al*., 2014). Solano-López and Hernández-Sánchez (2000) studied the behaviour of *L. monocytogenes* during the manufacture and ripening of two Mexican cheeses. They found that *Listeria monocytogenes* was able to survive the manufacture and ripening processes of both Mexican cheeses. However, Ortenzi *et al.* (2015) artificially contaminated raw milk used for producing cheese with *L. monocytogenes* and realized that there was a significant decrease of the viable counts of *L. monocytogenes* during the ripening and storage of the cheese. This suggested that the cheese could not support the growth of *L. monocytogenes.*

2.17 Dose -Response: factors affecting dose-response in relation to *Listeria*

The response of an individual to exposure to food-borne pathogens depends on several factors such as the pathogen virulence characteristics, the number of cells ingested, the general health and immune status of the host and any other thing in the food that alter the host status. Hence for any individual to become ill as a result of exposure to a food-borne pathogen, it will depend on the integration of host, pathogen and food matrix effects (FAO, 2004). These classes of factors are discussed below.

2.17.1 The virulence pathogen

The species of the genus *Listeria* that are capable of producing listeriolysin are those capable of causing diseases. Listeriolysin is a haemolysin (that is, an enzyme capable of lysing red blood cells) produced by *L. monocytogenes* and is associated with the ability's of the bacteria to cause disease. All *L. monocytogenes* are considered to be pathogenic, however there exist differences in relation to the virulence of individual strains (Hof and Rocourt, 1992). Thus, the number of microorganisms required to produce an infection, the potential for an infection to become symptomatic, the severity or manifestations of illness, and which individuals in the population are at greatest risk and the likelihood of reaching the intestinal tract are influenced by the variability of the strain/isolates of *L. monocytogenes* (FAO, 2004).

2.17.2 Host susceptibility

The response to infectious agents in human populations are quite different, showing the population's diversity in genetic background, general health and nutritional status, age, immune status, stress level and prior exposure to infectious agents. *L. monocytogenes* posseses mechanisms for avoiding or overcoming host's defences. Thus prior exposure to *L. monocytogenes* by an individual does not render the person resistant to subsequent exposures to the pathogen.

Individuals who have an underlying condition that suppresses their T-cell mediated immunity are those normally affected by severe listeriosis (Farber and Peterkin, 1991; Rocourt, 1996). Individuals with such immunity are thus at risk and they include elderly and the neonates and pregnancy, risk factors include cancer and immunosuppressive therapy, AIDS, and chronic conditions such as cardiovascular disease, congestive heart failure, diabetes, cirrhosis and alcoholism (Goulet and Marchetti, 1996; Rocourt, 1996).

2.17.3 The Food matrix

There has been an increasing awareness of the fact that the food matrix can contribute to the likelihood of diseases in humans. *Listeria* can survive the pH of gastric fluids thus surviving the first body's defence against it (O'Driscoll *et al*., 1996). Foods with high buffering capacity may also protect the pathogen from gastric acid, although the gastric response to exogenous buffers may be complex (Blaser and Newman, 1982). Foods with high fat contents also protect the *Listeria* from the gastric acid during passage through the stomach (Blaser and Newman, 1982).

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study design

The study was designed in two parts. First, a survey was conducted on the dairy sector in the study area to assess background information of the practices in the traditional dairy industry and to determine the technology and knowledge used in the production of raw milk and milk products using questionnaire. The second part was laboratory analysis to determine the prevalence of *Listeria* species and *L. monocytogenes* in samples of raw milk and milk products obtained from the study area.

3.2 Study area

The study was carried out in four Local Government areas of the Northern Region of Ghana. These local Government areas studied include Tamale Metropolis, Savelugu/Nanton, Central Gonja and East Gonja (Figure 3.1). Northern region is located in the northern part of Ghana and it is currently divided into twenty six (26) Local Government areas (Northern, 2013). This region has a very large number of dairy farms with high cattle population. The ecology of the place favours cattle rearing since it is a grassland. Some of these local government areas do not have fresh vegetation throughout the year.

The choice of these four local Government areas was based on the population distribution of dairy farms and for that matter cattle population. There is an agricultural establishment like National Veterinary College at Pong-Tamale and other dairy farms within the study area.

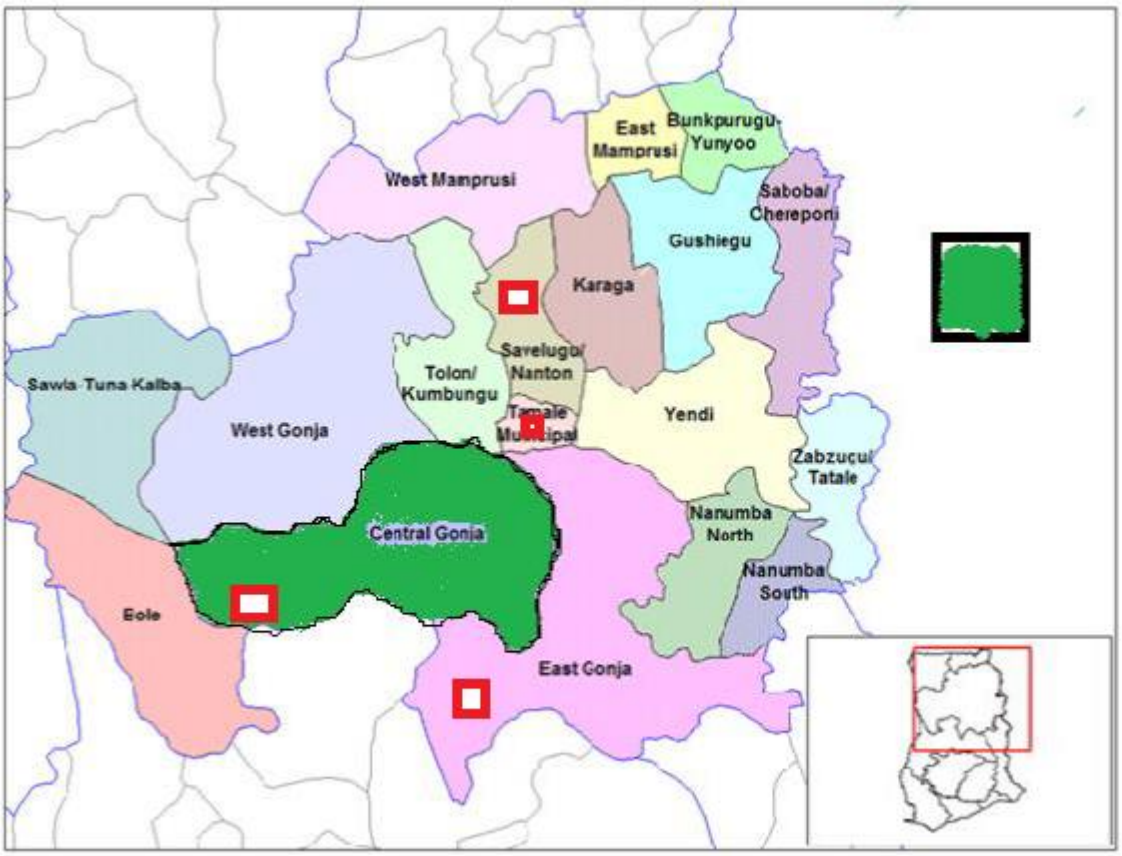

Figure 3.1: Map of the Northern Region and its Districts, including the four in which samples were taken

Source: GSS (2010)

3.3 Questionnaire design and administration

A questionnaire based pre-survey was designed and administered randomly to dairy farm owners about the technology and knowledge in the dairy value chain in the Northern Region. The selling lines and some factors that supposedly affect exposure to *Listeria* and *L. monocytogenes* such as management practice, milk marketing, processing, type of feed, storage, disinfection method, hygiene and sale of milk and many others were investigated. A total of 85 questionnaires were administered to 85 dairy farm owners, producers and processors about management practice, milk production, milk and milk product marketing and processing. These categories of people within the dairy value chain were selected as a result of a purposive survey

carried out in some administrative areas to determine the availability of dairy farms, producers and processors. Sampling was therefore purposive and random.

3.4 Laboratory investigations

3.4.1 Sampling of milk and milk products

Samples of milk and milk products were collected from the four (4) administrative areas including Tamale, Savelugu/Nanton, Central Gonja and East Gonja in the Northern region of Ghana. The details of the administrative areas together with the number of samples collected are presented in Table 3.1

Table 3.1: The administrative areas and total number of samples collected from them

Administrative area	Number of samples
Tamale	41
Savelugu/Nanton	40
Central Gonja	41
East Gonja	41

A total of 163 samples including 57 raw milk from dairy farm, 28 pasteurized retail milk, 27 cheese, 25 spontaneously fermented milk, 17 starter culture fermented yoghurt and 9 fried cheese were collected randomly from dairy farms, street hawkers, markets and producers for laboratory analysis. Samples were aseptically collected into individual sterile bags and further transferred into a zip locked bag, kept in ice chest containing ice packs and immediately transported to the Biotechnology laboratory of the Savanna Agriculture Research Institute (SARI), Nyankpala where they were analyzed immediately. Sampling and laboratory analysis were carried out from July, 2015- January, 2016.

3.4.2 Isolation of *Listeria* species on selective agar media

Techniques for the isolation of *Listeria* on selective agar were according to the methods described by Hitchins (2001) and Roberts *et al*. (1995). All the media used in this study were obtained from oxoid (Oxoid, 2006). Twenty five (25) ml or g of each dairy sample was aseptically added to 225 ml of 1% buffered peptone water (BPW) and homogenized in a blender for 1 minute for thorough mixing. The homogenate was further incubated at 30°C for 24 h. Ten ml of the homogenate was representatively added to 90 ml listeria enrichment broth base (CM 0862, Oxoid) with selective enrichment supplement (SR 0141, oxoid) containing nalidixic acid, cyclohexemide and acriflavine hydrochloride and incubated at 30°C for 48 h. A 0.1ml of homogenate was surface plated in duplicate on Palcam agar (CM0877, Oxoid) supplemented with Palcam selective supplement (SR0150E, Oxoid) and Oxford agar (CM0856, Oxoid) plates with listeria selective supplement (SR0140, Oxoid) respectively. Plates of the palcam (polymyxin acriflavin lithium-chloride ceftazidime aesculin mannitol) and Oxford were incubated at 37°C for 48 h under microaerophilic conditions. After 48 hours of incubation, the presumptive *Listeria* species formed colonies that appear to be approximately 2 mm in diameter, grey green in colour with a black sunken centre and a black halo against a cherry-red medium background.

3.4.3 Biochemical characterization

About five suspected *Listeria* colonies were picked from each selective agar plate and transferred onto Tryptone soya agar (CM0131, oxoid) to be purified before being subjected to the Grams and catalase reactions.

3.4.3.1 KOH solubility test

For the determination of Grams reaction, one drop of 3% KOH was placed on a clean dry glass slide. A colony of growth from the tryptone soya agar was picked with a sterile loop and added to the drop of the 3% KOH aqueous solution and mixed well with the loop. The

loop was then raised a few centimeters from the glass slide by touching the mixture. It was observed for viscous or mucoid strand.The production of mucous or mucoid strand was an indicative of gram negative bacteria (Halebian *et al*., 1981)

3.4.3.2 Catalase test

One drop of 3% hydrogen peroxide was placed on a clean grease glass slide. A colony of growth from the tryptone soya agar was picked with a sterile toothpick and placed on the drop of the 3% hydrogen peroxide. This was observed for bubbles. The result was recorded accordingly. The production of bubbles confirms the presence of aerobic and facultative anaerobic bacteria. An enzyme called "catalase" was produced by the bacteria and this enzyme breaks down the hydrogen peroxide into water and oxygen (Chwuku, 2007). The release of oxygen is an indicative of the bubbles produced. Chemically, the reaction is given below: Colony + $2H_2O_2 \rightarrow 2H_2O + O_2$ (Roberts and Greenwood, 2003; Chwuku, 2007)

3.4.5 Polymerase Chain Reaction (PCR) detection of *Listeria* species and *L. monocytogenes*

3.4.5.1. Colony PCR method

Following biochemical testing, the presumptive isolates were subjected to PCR analysis to ascertain the presence of *Listeria* and *L. monocytogenes*. Presumptive positive isolates were screened for the presence of 16S rRNA gene in *Listeria* and listeriolysin O (*hlyA*) gene in *L. monocytogenes*. The primer sequences were U1 ([5'- CAGCMGCCGCGGTAATC-3']) and LI1 [5′-CTCCATAAAGGTGACCCT-3′] for all *Listeria* species, and LM1 (5'-CCT AAG ACG CCA ATC GAA-3') and LM2 (5'- AAG CGC TTG CAA CTG CTC-3') for *L. monocytogenes*. DNA was extracted by the thermal lysis method as in AL-Ashmawy *et al* (2013) before PCR was undertaken. To extract the DNA, a single colony from the tryptone soya agar plate was grown on oxford agar and palcam agar plates respectively. These were incubated for 48 h at 30 °C. From each plate, 3-5 colonies were randomly selected using a

sterile toothpick and suspended in a 1.5 ml eppendorf tube containing 50 µl of sterile distilled water. This was incubated for 5 min at 90 °C. This was then centrifuged at 13 000 rpm for 1 min. The supernatant was taken and transferred into a separate 0.5 ml eppendorf tube. An aliquot of 1 µl of the supernatant was used as template for PCR. The PCR was performed in a final volume of 10 µl containing 5 µl OneTaq 2X Master Mix with standard buffer, 1 µl primer, 1 µl DNA and 3 µl Nuclease free water. The cycling conditions consisted of initial denaturation of 95^{o}C for 4 min, followed by denaturation of 30 cycles at 95^{o}C for 1 min, annealing, 52^{o}C for 45s for 30 cycles, extension, 72°C for 2 min and final extension of 30 cycles at 72^{o}C for 8 min. The PCR products were checked and separated in 1.2% agarose gel and stained with ethidium bromide. The separated PCR products were visualized using the UV Trans-illuminator.

3.4.5.2 Enrichment PCR method

DNA was first extracted by thermal cell lysis of suspended bacteria from the enrichment broth. Following the enrichment of milk (25ml) and milk products (25g each) in 225ml of 1% BPW at 30^{o}C for 24 h, 15 ml of the enrichment sample was transferred into ten 1.5 ml eppendorf tubes. These were centrifuged for 10 min at 4000 rpm and the supernatants discarded. The harvested cell pellets left after discarding the supernatants were re-suspended in 1 ml sterile distilled water and centrifuged at 14, 000 rpm for 10 min after vortexing. The supernatants again were carefully discarded and the pellets were re-suspended in 100 µl of sterile distilled water. They were vortex and centrifuged at 14, 000 rpm for 10 min and the supernatants discarded carefully. The pellets were again re-suspended in 100 µl sterile distilled water, vortexed and incubated at 95^{o}C for 15 min. The cell debris formed after incubation was pelleted by centrifugation at 14,000 rpm for 10 min. The DNA present in the supernatants of the ten eppendorf tubes was then transferred into a 0.5 ml eppendorf tube and stored at -20°C until PCR analysis was carried out. An aliquot of 1 µl of the supernatant was

used as the template DNA. The template DNA was used for PCR analysis to detect 16S rRNA and listeriolysin O (*hlyA*) genes using the primer set U1 ([5'-CAGCMGCCGCGGTAATC-3']) and LI1 [5′-CTCCATAAAGGTGACCCT-3′] for *Listeria* species and primer set LM1 (5'-CCT AAG ACG CCA ATC GAA-3') and LM2 (5'- AAG CGC TTG CAA CTG CTC-3') for *L. monocytogenes* (Border *et al*., 1990; AL-Ashmawy *et al.*, 2013; Eruteya *et al.*, 2014). The 16S rRNA is a highly conserved gene in all *Listeria* species and the LI1 primer is complementary to it. The *hlyA* gene sequence in *L. monocytogenes* was detected using the primers LM1 and LM2. The PCR was performed in a final volume of 10 µl containing 5 µl OneTaq 2X Master Mix with standard buffer, 1 µl primer, 1 µl DNA and 3 µl Nuclease free water. The PCR conditions consisted of initial denaturation of 95°C for 4 min and followed by denaturation 30 cycles of 95°C for 1 min, annealing, 52 °C of 45 s and extension of 72 °C for 2 min and final extension of 72°C for 8 min .The U1/LI1 primers amplified 938 bp of all the *Listeria* species in the region 16S rRNA sequence. The LM1/LM2 primers amplified approximately 702 bp of *L. monocytogenes* in the *hlyA* gene.

3.4.6 Determination of the performance of PALCAM agar and OXFORD agar in detecting *Listeria* species

The performance of these two media was determined by considering the presumptive, biochemical and PCR results. Representatively, 3-5 presumptive positive colonies were initially confirmed by biochemical analysis prior to PCR analysis. The detection and confirmation of *Listeria* in a sample using any of the media scored such a sample as positive for *Listeria. Listeria* presumptive colonies from any sample detected on any media makes the sample presumptively "positive" based on that particular media, and if there was no colony growth then it was considered "negative". Any presumptive positive sample from the media that failed to be confirmed by PCR as *Listeria* was considered as "false positive". Similarly,

any media that fail to detect *Listeria* in a *Listeria*-positive sample whether at the presumptive level or after confirmation was considered as "false negative" (Jamali *et al*., 2012). Based on the criteria described by Willinger and Manafi (1999), the following values were calculated: sensitivity (%) = true positive × 100/(true positives + false negatives), specificity (%) = true negatives × 100/(true negatives + false positives), and efficiency (%) = (true positives + true negatives) × 100/ total

3.5 Statistical Analysis

Data obtained from the survey and the frequency of isolating *Listeria* species were presented using descriptive statistics. Data on the effectiveness of the various types of PCR, and the relationship between the true/false positive results and the food categories were analyzed using chi-square analysis. The statistical analyses were all performed with SPSS version 15 software. Differences were considered significant at values of $P<0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 Characteristics of study population

A total of 85 randomly selected dairy farmers and producers of milk products were interviewed from the four administrative areas comprising of Tamale Metropolis, Savelugu Municipal, Central Gonja District and East Gonja District all in the Northern Region of Ghana. Of the 85 dairy farm owners and processors, 27 were milk producers only, 52 were both milk producers and processors and 6 were processors only. Sex and age characteristics of respondents are as shown in Table 4.1.

Table 4.1 Demographic characteristics of milk producers and processors

Demographic characteristics	Number (%)
Sex	
Male	48 (56.5)
Female	37 (43.5)
Age characteristics	
≤ 20	2 (2.4)
21 – 30	3 (3.5)
31 – 40	9 (10.6)
41 – 50	18 (21.2)
51 – 60	40 (47.1)
≥ 61	13 (15.3)
Education	
None	79 (92.9)
Basic	2 (2.4)
Secondary/Technical/Vocational	4 (4.7)
Tertiary	0 (0.0)

The data on the technology and knowledge in the dairy value chain in the Northern Region includes management practice, milking production, processing, disinfection method, hygiene and sale of milk. The respondents were dairy farmers, producers and processors. None (92.9%) of the respondents who are dairy farmers had any formal education. However, about 7.1% of the processors had formal education. The following sections describe the milk production process, milk processing, sale and management practices.

4.2 Milk production and processing

4.2.1 Milk production

Milking of cows in the Northern Region is done manually by hand. The milking of cows is done both at mornings and evenings. Information given by producers indicates that, 88.6% usually milk once in a day while 11.4% milk twice in a day depending on the season. The Northern Region of Ghana do not experience rains throughout the year hence there are two seasons; Rainy and Dry seasons. It is during the rainy season that some (11.4%) producers usually milk twice in a day. In some parts of the Region, there is no milking during the dry season. The milking and storage containers used during milk production are mainly calabashes, plastic containers and silver/aluminium bowls. According to the information gathered from the respondents, the average milk produced per day is 14 litres during the rainy season and 8 litres during the dry season, from average cattle of thirteen per day. Cleaning/disinfection of cattle udder before milking is not commonly practice in the study area. Majority (70.9%) of farmers neither washes nor disinfects their hands during milking. However, all respondents (100%) indicated that milking containers were washed with water and soap. Actors in the milk value chain including dairy farmers, producers and processors of milk do not keep records (bookkeeping) of their business activities.

4.2.2 Milk processing

Processing of milk into various milk products is a common practice among the people in the Northern Region of Ghana. Fresh milk remains the starting point for these milk products. The conversion of milk into milk products are mainly for preservation and the conservation of the milk nutrients. Natural fermentation, commercial starter culture and addition of *Calotropis procera* juice are the means employed in producing the milk products. Fresh milk is used to make a number of milk products in the Northern Region of Ghana. The milk products commonly made by producers include soft cheese, yoghurt and butter. Detailed description of the production process of the common milk products are shown in Figure 4.1

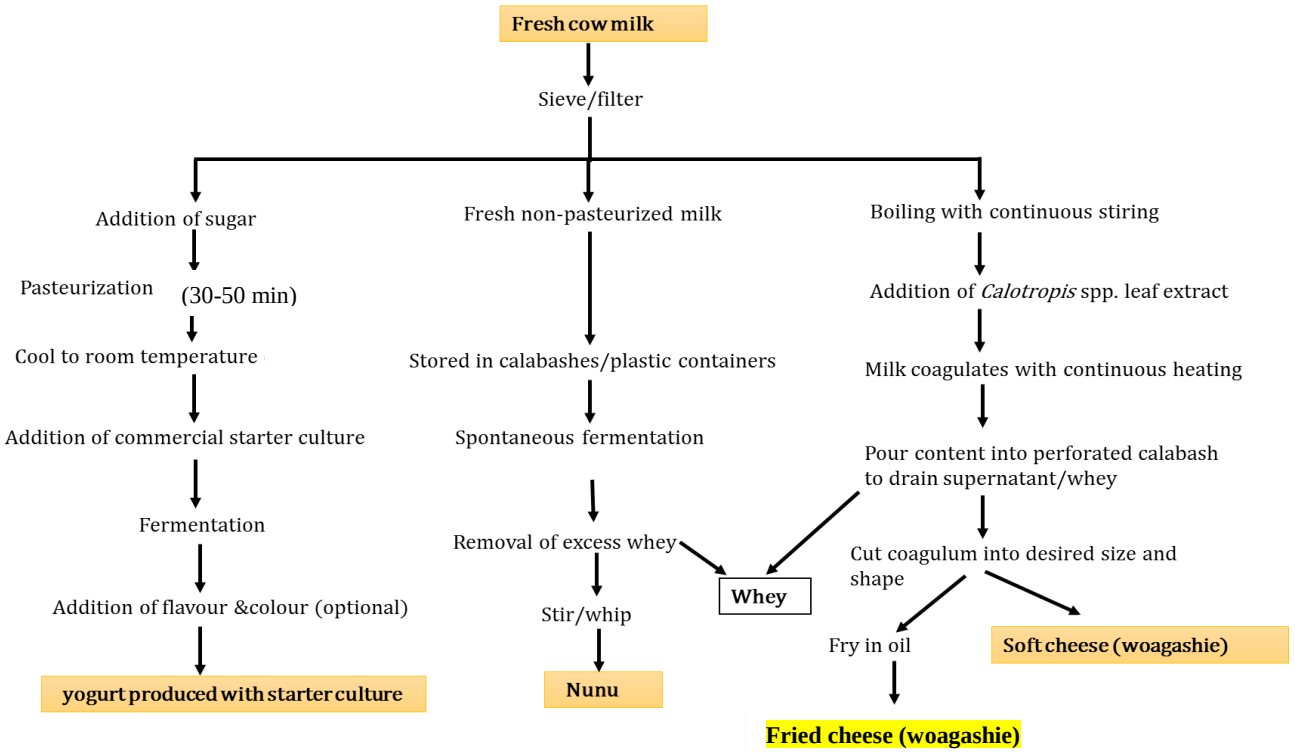

Figure 4.1: Traditional processing of fresh cow milk into various traditional products in Northern Region-Ghana

4.2.3 Sale of milk

In the Northern Region of Ghana, all (100%) milk producers interviewed usually sieve milk to remove debris and particles of fur before selling it to the public or processing. The milk may be sold to the public either raw or boiled (pasteurized). Milk intended to be sold directly to consumers is boiled by the producers. From the respondents who sieved and boiled or pasteurized their milk, 77.2% of them mentioned that they do so in order to remove dirt, kill germs and to let the milk stay fresh for long/prevent coagulation, while 22.8% mentioned that they practice this only to remove dirt and kill germs. The milk processors middle sales persons 26.6% (21/79) buy milk at the dairy farm and transport to market for sale or process them into various products such as fermented milk (*nunu*) or *woagashie*. The middle sales persons pool together milk purchased from several producers, and then sell it to vendors. It is important to state that the middle sales persons in this study equally produce milk. They sell the milk in large volumes to milk vendors, who may process it into other products. It is however important to state that 76.2% (16/21) of middle sales persons in this study processed the milk into milk products while 23.8% (5/21) of them sell raw milk to vendors. Information obtained from the producers indicated that, 65.8% (52/79), 34.2% (27/79) and 0.0% (0/79) of them sell milk and milk products, only milk and only milk products respectively. There is no dedicated market place solely meant for the sale of milk and milk products alone. Fresh milk and milk products can be purchased at various dairy farms, main market, mini sales point, and other vendors who carry the milk round the city, town or village on heads. Different means of transport are used to transport milk to market, depending on the distance. In this study, 68.9% (42/61) of producers who sell milk and milk product directly to consumers carry them on heads, while 21.3% (13/61) and 9.8% (6/61) of producers transport the milk to market or consumers by means of cycles and market trucks respectively. The choice of transport depends largely on the distance to the sales point and availability of resources either

to purchase a cycle or board a truck. The time spent to transport pasteurized milk from production site to consumers varies between less than 30 minutes and more than one hour. From the 61 respondents who sell milk directly to consumers, 46 (75.4%), 9 (14.8%), and 6 (9.8%) of them spent 30-60 minutes, less than 30 minutes and more than 60 minutes respectively to transport milk to the consumers. None of the milk vendors and producers disinfects, wears gloves or washes their hands with soap whiles handling and selling milk to consumers. The selling point of milk to consumers varies. It is common to see vendors selling milk to consumers by hawking village or town, sitting at a special milk sale point or at the general open market. In this study, 26 (42.6%), 16 (26.2%) and 19 (31.1%) of the respondents are hawking, sit in the open markets and sit at the milk sale points respectively to sell fresh milk and milk products. The details of the results are shown in Figure 4.2 below

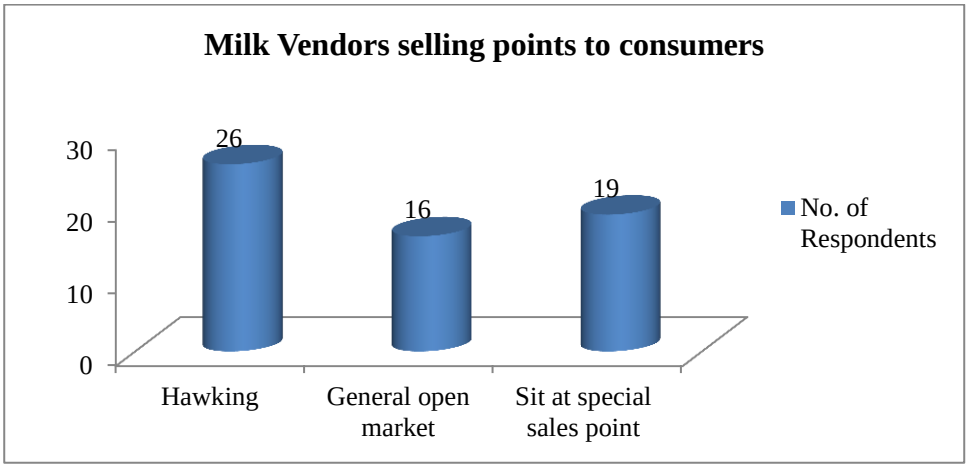

Figure 4.2: Selling points of milk by milk vendors

4.2.4 Management practice

A total of 79 randomly selected farmers who produces milk were interviewed on management practice of cattle herds. From these 79 respondents, 67% of them fenced their cattle while 33% kept their cattle in open space upon the return of the cattle from the field. All respondents (100%) indicated that tethering is commonly practiced for calves and milking

cattle. All (100%) the cattle farmers feed their cattle by means of free range system. In the Northern Region of Ghana, 88.6% cattle farmers have access to veterinary services. Information obtained from the respondents indicated that, 48.6% (34/70), 7.1% (5/70), and 44.3 % (31/70) receive veterinary services only treatment when cattle are sick, for both vaccination and treatment respectively and regularly for diagnoses. The detailes of the results are shown in Figure 4.3 below

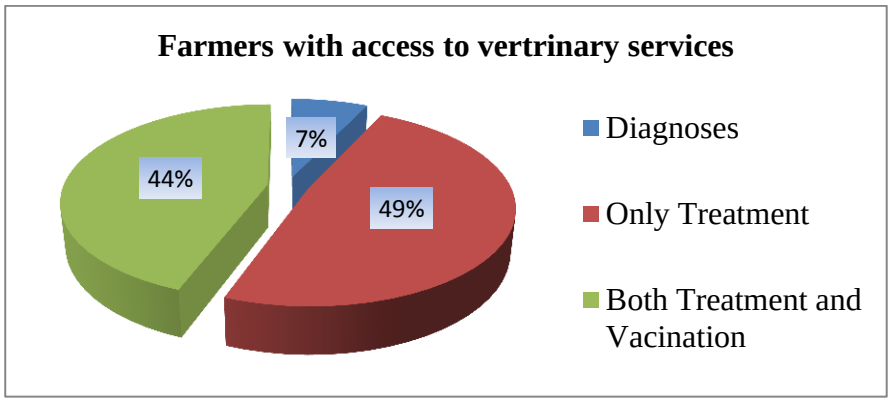

Figure 4.3: Service type received by Farmers from the Veterinary Officers

About 80%, 60%, 37%, 60%, 6.3%, and 4% of the respondents indicated that diarrhea, weakness, pink eye infection, mastitis and abortion respectively are the common clinical signs observed in their herds. Nineteen (24.1%), 14(17.7%), 10(13%), 7(8.9%), 6(7.6%) and 0(0.0%) of the respondents indicated that contageous bovine pleura pneumonia, black quarter, salmonellosis, tuberculosis, brucellosis and anthrax, respectively are the most important diseases among cattle. About 23 (29.1%) could not describe the kinds of diseases attacking their cattle herds. Of the 79 farmers interviewed, 31 (39.2%) of them indicated that their cattle herds are regularly vaccinated against contageous bovine pleura pneumonia, anthrax, brucellosis and black quarter while 48 (60.8%) indicated that they do not vaccinate their cattle against any disease.

4.3 Detection of *Listeria* species

4.3.1 Detection of *Listeria* species in milk and milk products

Listeria species was isolated from 34/163 (20.9%) and 38/163 (23.3%) of raw milk and dairy product samples based on growth on oxford and palcam selective media respectively. The prevalence of *Listeria* in raw milk samples obtained from dairy farms was higher than that of the pasteurized milk obtained from street and market vendors. With regard to the other dairy products, the soft cheese recorded the highest percentage with fried cheese and yoghurt with starter culture being the least on both oxford and palcam agar plates. Table 4.2 shows the prevalence of *Listeria* species in all the milk and milk products samples.

A **B**

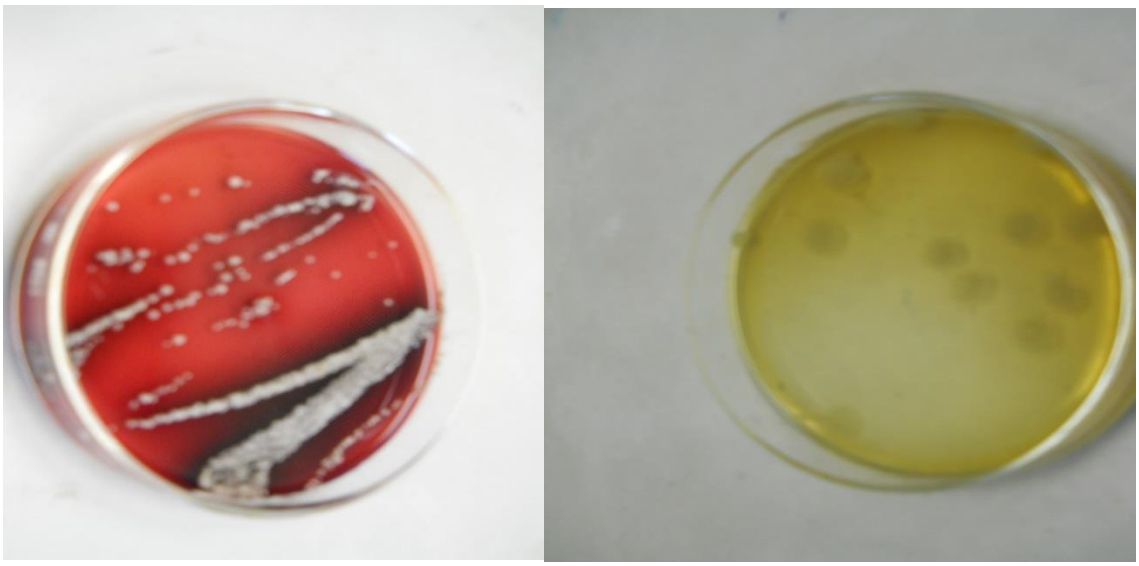

Figure 4.4: A 48 h culture of *Listeria* species on different selective agar media. **A:** Colonies of suspected *Listeria* specie on palcam agar media **B:** Colonies of suspected *Listeria* species on oxford agar media

Table 4.2: Prevalence of *Listeria* species in milk and milk products using different agar media

Milk sample/Product	Number of samples (n)	Listeria (genus) positive (%)	
		Oxford agar	Palcam agar
Raw Milk (RM)	57	12(21.1)	13(22.8)
Pasteurized Milk on Market (PMM)	28	3(10.7)	3(10.7)
Soft Cheese (SC)	27	12(44.4)	12(44.4)
Fried Cheese (FC)	9	2(22.2)	2(22.2)
Yoghurt with Starter Culture (YSC)	17	1 (5.9)	3(17.6)
Spontaneously Fermented Milk (SFM)	25	4(16.0)	5(20.0)

4.3.2 Confirmation of *Listeria* species by biochemical methods

The biochemical examination of isolates using the catalase and gram reaction tests indicated that 64.7% (110/170) of all the tested positive colonies on oxford agar were positive for *Listeria* species. Similarly, the biochemical test for positive *Listeria* isolates on palcam agar was 57.9% (110/190) for all milk and milk products. Table 4.3 shows the number of *Listeria* isolates confirmed by biochemical methods.

Table 4.3: Confirmation of *Listeria* species by biochemical methods

	OXFORD AGAR		PALCAM AGAR	
Milk sample/product	Total number of colonies	Gram+/Catalase + (%)	Total number of colonies	Gram+/Catalase + (%)
RM	60	40(66.7)	65	41(63.1)
PMM	15	7(46.7)	15	12(80.0)
SC	60	46(76.7)	60	36(60.0)
FC	10	0(0.0)	10	0(0.0)
YSC	5	3(60.0)	15	8(53.3)
SFM	20	14(70.0)	25	13(52.0)

RM-Raw Milk, PMM- Pasteurized Milk on Market, SC- Soft Cheese, FC- Fried Cheese, YSC- Yoghurt with Starter Culture, SFM- Spontaneously Fermented Milk

4.3.3 Detection of *Listeria* and *Listeria monocytogenes* in raw milk and milk products using colony PCR

Colony PCR confirmed 87 (39.5%) out of the 220 suspected *Listeria* colonies on the selective agars isolated by cultural methods from the samples of milk and milk products as *Listeria* species having the conserved gene, 16S rRNA, using the primer pair U1 ([5'-CAGCMGCCGCGGTAATC-3']) and LI1 [5′-CTCCATAAAGGTGACCCT-3′] (Figure 4.5). Table 4.4 shows that *Listeria* species was not detected in pasteurized milk but detected in fresh milk obtained from dairy farms. *Listeria* species was detected in all milk products except fried cheese, pasteurized milk and yoghurt produced with starter culture. The primers LM1 (5'-CCT AAG ACG CCA ATC GAA-3') and LM2 (5'- AAG CGC TTG CAA CTG CTC-3') were used for the detection of the virulence factor, Listeriolysin O (LLO), associated with *L. monocytogenes*. The virulence factor was encoded by the *hlyA* gene in *L. monocytogenes*. The colony PCR also identified 30 (34.5%) out of the 87 colonies confirmed as *Listeria* species by colony PCR as having the *hlyA* gene of *L. monocytogenes* (Figure 4.6).

L. monocytogenes was frequently detected in raw milk obtained from dairy farms but was not found in pasteurized/boiled milk obtained from market and street vendors. Among the milk products *L. monocytogenes* was frequently isolated from soft cheese and less frequently in spontaneously fermented milk as shown in Table 4.4. *L. monocytogenes* was not detected in yoghurt produced with starter culture, pasteurized milk and fried cheese. There was a mismatched of the primers in the position for the *L. monocytogenes.* The forward primer was homologous to the reference sequence of *L. monocytogenes* considered. However, the reverse primer amplified a region of approximately 852 bp also corresponding to the *hlyA* gene of *L. monocytogenes* as in Montero *et al*. (2015). This indicated that both the forward and reverse primers are able to amplify the desired gene (*hlyA*) of *L. monocytogenes.*

Table 4.4: *Listeria* species and *Listeria monocytogenes* detected by colony PCR and Enrichment PCR

	Enrichment PCR			Colony PCR		
Milk sample/product	Number of samples	Number of + samples for *Listeria* spp.	Number of + samples for *L. monocytogenes*	Number of colonies	Number of +coloniesfor *Listeria spp.*	Number of + colonies for *L. monocytogenes*
RM	57	10	5	81	38	13
PMM	28	0	0	19	0	0
SC	27	9	5	82	39	15
FC	9	0	0	0	0	0
YSC	17	0	0	11	0	0
SFM	25	3	1	27	10	2

RM-Raw Milk, PMM- Pasteurized Milk on Market, SC- Soft Cheese, FC- Fried Cheese, YSC- Yoghurt with Starter Culture, SFY- Spontaneously Fermented Milk

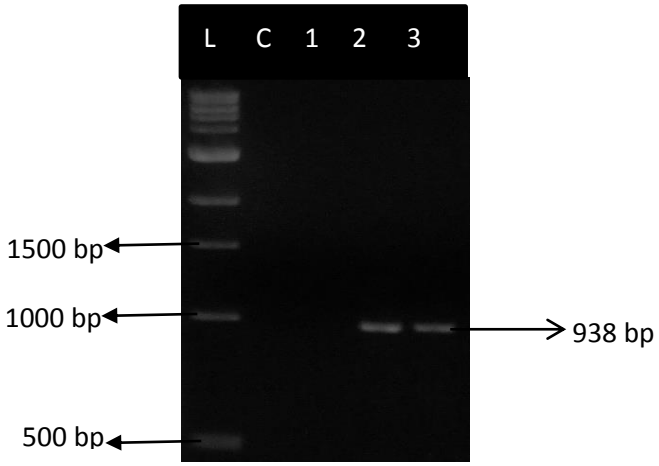

Figure 4.5: An amplified Colony PCR product of *Listeria* species indicated by a single band at 938 bp. The primer LI1 and U1 combination were used. Lane L, 1 kb ladder, C, negative control (nuclease free water), 1 to 3 are samples of milk and milk products containing DNA

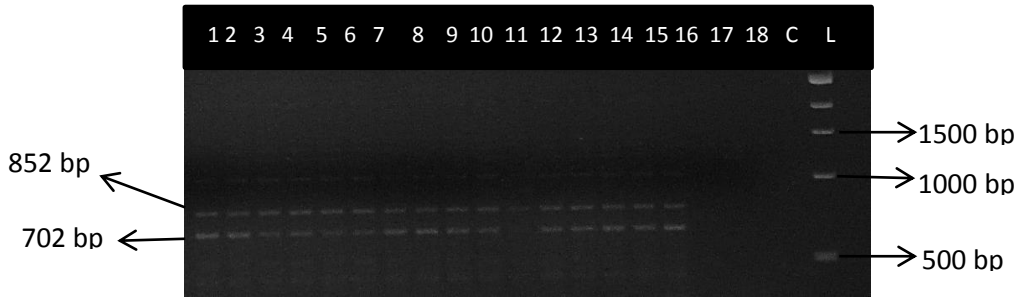

Figure 4.6: An amplified ColonyPCR product of *L. monocytogenes* indicated by a single band at 702 bp. The primer LM1 and LM2 combination was used. Lane L, 1 kb ladder, 1 to 18 indicates the samples of milk and milk products containing DNA, C, negative control (nuclease free water). The same primer set produced another band at 852 bp which also correspond to the Listeriolysin O (*hlyA*) of *L. monocytogenes*.

4.3.4 Detection of *Listeria* species and *L. monocytogene* using Enrichment PCR technique

The enrichment PCR identified the presence of 16S rRNA of *Listeria* species in 33.3%, 17.5% and 11.5% of soft cheese, raw milk and spontaneously fermented milk respectively (Figure 4.7), but not detected in pasteurized milk, fried cheese and yoghurt produced with starter culture. The *hlyA* gene used to detect the presence of *L. monocytogenes* indicated the prevalence of 18.5%, 8.8% and 4% detection rates in soft cheese, raw milk and spontaneously

fermented milk respectively. No *L. monocytogenes* was detected in pasteurized milk, fried cheese and yoghurt produced with starter cultre.

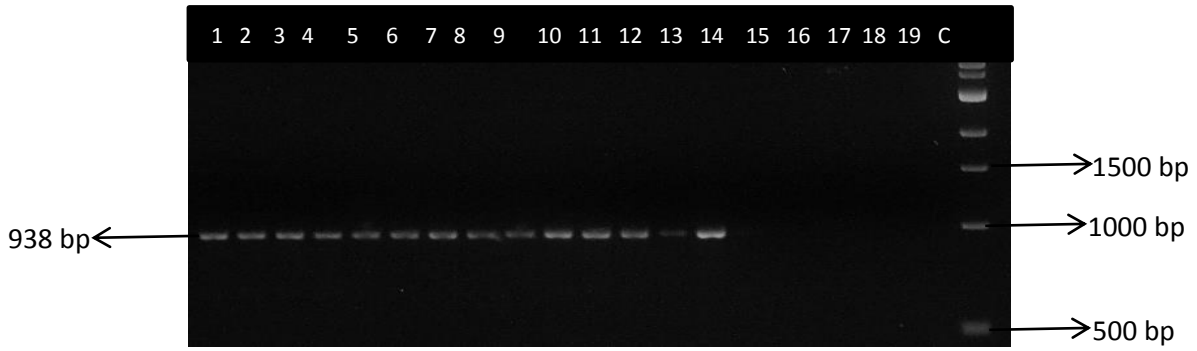

Fig 4.7: An amplified Enrichment PCR product of *Listeria* species indicated by a single band at 938 bp. The primer LI1 and U1 combination was used. Lane L, 1 kb ladder, C, negative control (nuclease free water), 1 to 19 are samples of milk and milk products containing DNA

4.3.5 Comparison of Enrichment and Colony PCR methods for the detection of *Listeria* species and *L. monocytogenes* in milk and milk products

The overall occurrence of *Listeria* species and *L. monocytogenes* in the samples determined by enrichment PCR were 22/163 (13.5%) and 11/163 (6.7%) respectively. Out of the 87 *Listeria* colonies from 21/163 (12.9%) samples, 30 were confirmed by colony PCR as *L. monocytogenes* from 11/163 (6.7%) samples. Thus, both colony PCR and Enrichment PCR techniques detected the same prevalence for *L. monocytogenes*. While colony PCR detected 21 *Listeria* contaminated samples, enrichment PCR detected 22 *Listeria* contaminated samples. The difference in performance efficiency between Enrichment PCR and Colony PCR was not statistically significant ($p>0.05$) (Appendix 4 Table 3).

4.3.6 Comparing the efficiencies of Oxford and Palcam agar in detecting *Listeria* species

Two plating media comprising of Oxford and Palcam were compared for their efficiency in detecting *Listeria* species. The process of detecting using such media took 48 h. There was a difference in efficiency between the two plating media but not more than 3.7%. Oxford agar recorded the highest efficiency of 91.4% while palcam recorded 87.7%, however there was no significant difference between the rates of detection of *Listeria* species using the two agar media in terms of their efficiencies (P>0.05) (Appendix 4 Table 1) . The plating media were highly sensitive in detecting positive samples with Oxford agar again recording the highest percentage as shown in Table 4.5. The detection of false negatives was decreased by 50% by Oxford agar as compared to palcam agar. Statistically there was no significant relationship between the true/false positive results and the food categories (P>0.05) (Appendix 4 Table 2). All false positive colonies on oxford agar and palcam agar were suspected to be other gram positive, catalase positive bacteria. These two media allowed more than one step detection of *Listeria* species as further biochemical and PCR was required.

Table 4.5: Specificity, sensitivity and efficiency of palcam and oxford agar based on the 163 samples

Media	True positive	False positive	True negative	False negative	Sensitivity (%)	Specificity (%)	Efficiency (%)
Palcam	20	18	123	2	90.9	87.2	87.7
oxford	21	13	128	1	95.5	90.9	91.4

Sensitivity = True positives × 100/(True positives + False negatives)
Specificity = True negatives × 100/(True negatives + False positives)
Efficiency = (True positives + True negatives) ×100/Total

CHAPTER FIVE

5.0 DISCUSSION

5.1 Management of cattle farms, milk production and procession

The study showed that health of cattle farms is a common practice in the study area, done primarily to ensure that cattle herds are healthy in order to produce quality milk. The results of the common clinical signs in cattle in this study are consistent with that of Hempen *et al.* (2004) who reported similar clinical signs in cattle herds in Guinea, Gambia and Senegal. Normally, free grazing of the cattle as indicated by the respondents predisposes the cattle to infections such as *L. monocytogenes,* subsequently, leading to listeriosis. Chukwu (2007) reported that *Listeria* species and *L. monocytogenes* have been isolated from green vegetation, water sources, soil, and human and domestic animals faeces hence the practice of free grazing exposes cattle to such contamination since they frequently consume them.

The habit of not cleaning and disinfecting udders and teats of milking cows is consistent with that of Hempen *et al.* (2004), who reported similar practice in Gambia and Guinea. Also the practice of milking once per day in the study area is equally done in Gambia and Guinea, and that of milking twice is also practice in Tambacouda in Senegal (Hempen *et al.*, 2004). The habit of using aluminium bowls, calabashes and plastic containers in handling milk by the respondents in this study is consistent with Omore *et al.* (2003) who reported that plastic buckets/basins, plastic gallons, jerry cans, milk cans/churns, aluminium basins/bowls and calabashes are used in handling milk in Kumasi and Accra in Ghana. The pooling of milk from different producers by middle sales persons is consistent with that reported by Hempen *et al.*, 2011; Omore *et al.*, 2003). The average daily production of milk in the study area was 8 litres and 14 litres in the dry and rainy (wet) seasons respectively. This indicates that, the quantity of milk produced in the rainy (wet) season is higher than the quantity produced in the dry season. This is consistent with Omore *et al.* (2003) who reported 10 litres in the dry

season and 12 litres in the wet season in Kumasi, and 20 litres in the dry season and 24 litres in the wet season in Accra. The results suggest that the availability of feed affect milk production. High quantity of milk produced during the wet season is due to the availability of more feed/forage.

The procession of milk into milk products is commonly done in the study area. Natural /spontaneous fermentation and *Calotropis procera* juice as indicated by the respondends are the means adopted by the processors during the production of fermented milk and soft cheese. The use of *Calotropis procera* in coagulating the milk during the soft cheese procession has been reported by Omore *et al.* (2003).

5.2 Isolation of *Listeria* species by culturing

The present study detected *Listeria* species in some samples of fresh milk and selected milk products tested after 24 h of enrichment, suggesting that a shorter enrichment time could be useful in detecting *Listeria*, instead of the 48 h recommended by most enrichment protocols. In this study, nonselective enrichment broth (BPW) was used which provided greater advantage in increasing the recovery of injured cells or the number of *Listerial* cells to detectable levels (Duffy *et. al.,* 2001; Law *et al*., 2015a). Hudson *et al.* (2001) indicated that isolation methods that do not use enrichment require more cells to be present before detection can be achieved. In addition, Gasanov *et al.* (2005) indicated that pre-enrichment helps to achieve the sensitivity of allowing one *Listeria* organism to grow to a detectable level of approximately $10^4 – 10^5$ CFU ml^{-1}.

In general Oxford agar was very effective than Palcam agar in detecting *Listeria* species. The biochemical studies of the various colonies on the agar plates were good for their tentative identification of *Listeria* even in the presence of other group of organisms. Nevertheless, based on the biochemical and the PCR results, some non –*Listerial* colonies were not

eliminated. There was no significant difference between the rate of isolation of *Listeria* species using oxford agar and palcam agar. Although the constituents of both media are slightly different, it did not bring out any significant difference in sensitivity, specificity and efficiencies. This result is in agreement with earlier reports by Chukwu (2007) who reported that there was no significant difference between between oxford agar and palcam agar in detecting *Listeria* species. This present study produced lower efficiencies of oxford and palcam agars in general as compared to Jamali *et al.* (2012). The efficiencies of Oxford agar and palcam agar in this study were 91.1% and 87.7% respectively which were lower than the 93.3% and 90.0% efficiencies of oxford and palcam reported by Jamali *et al.* (2012). The rate of isolation of *Listeria* species must have been influenced by the utilization of selective inhibitory constituents like lithium chloride, acriflavin, colistin sulphate, cefotetan, cycloheximide and fosfomycin and the indicators aesculin and ferrous iron in Oxford agar and Palcam agar (Gorski, 2008; Chukwu, 2007).

On oxford agar, *Listeria* species hydrolyzes aesculin to 6, 7 – dihydroxycoumarin producing black zones around the colonies due to the formation of complex with the ferric ammonium citrate which is a component of the media. But on the palcam agar, *Listeria* species appeared grey with black centres against a cherry red background colour. The *Listeria* colonies were almost visible by 24 h, but incubation was continued for 48 h to detect slow growing strains. The two plating media does not distinguish between *L. monocytogenes* from other *Listeria* species which have been noted to be non-pathogenic (El Marrakchi *et al.*, 2005).

5.3 Prevalence of *Listeria* species and *L. monocytogenes* in raw milk and milk products

The prevalence of *Listeria* species in soft cheese, raw milk and spontaneously fermented milk were 33.3%, 17.5% and 11.5% respectively. On the other hand, the prevalence of *L. monocytogenes* in soft cheese, raw milk and spontaneously fermented milk were 18.5%, 8.8% and 4% respectively. However, no *Listeria* species was detected in pasteurized milk, fried

cheese and yoghurt produced with starter culture sold on the markets. The observed difference in the prevalence rates of *Listeria* species and *L. monocytogenes* indicates that other species of *Listeria* which were not identified in this study also contaminate milk and milk products in the Northern region of Ghana.

The findings in this study are in close agreement with that reported by Jamali *et al.* (2013) and Seyoum *et al.* (2015) who reported a prevalence rate of 18.6% and 18.5% prevalence of *Listeria* species in raw milk. Meshref *et al.* (2015) in a study reported a higher prevalence of 27.45% *Listeria* in raw milk in Egypt whilst Moshoeshoe and Oliver (2012) and Alzubaidy *et al*. (2013) reported a lower prevalence of 4% and 9% of *Listeria* in non-pasteurised milk respectively. In a study by Kells and Gilmour (2004) a much higher incidence of 44.4% contamination of *Listeria* species was reported in raw milk samples in Northern Ireland. Our findings in this study are consistent with the results obtained by Vilar *et al.* (2007) who found *L. monocytogenes* in bulk milk samples at 6.1% and Gallegos *et al.* (2008) who found an incidence of 9.6% of *L. monocytogenes* in milk samples. Waak *et al.* (2002) and Aygun and Pehlivanlar (2006) reported that *L. monocytogenes* in raw milk samples were found to be 1% and 5% respectively. However, higher prevalence of *L. monocytogenes* (12.5%) has been reported by Holko *et al.* (2002). The implication of this finding is that raw milk obtained from dairy farms may serve as a direct source of *Listeria* infection. The samples were taken from milking sites, thus limit the influence by other factors such as filtering, cooling, storing or distributing milk. Nonetheless, *Listeria* is ubiquitous at the farms and this characteristic of *Listeria* has led to the detection of *Listeria* and *L. monocytogenes* in raw milk samples, since the pathogen is rarely excreted during the milking of cows even those with mastitis (Gallegos *et al.*, 2008). Bemrah *et al*. (1998) reported that the source of *Listeria* species in raw milk is attributed to faecal and environment contamination during milking, storage and transport, infected dairy animals and silage quality. In this study the contamination sources of fresh

milk obtained from dairy farms is probably insufficient hygiene during milking, ineffective cleaning of milking equipment, teats and udders of milking cows. It is imperative to note that silage is not used as feed for cows in Northern region.

The pasteurized milk samples investigated in this study were purchased from market vendors, street sellers and at sales point. The responses from the questionnaire administered indicate that milk sold to the public for consumption in Northern region is usually boiled or pasteurized. Neither *Listeria* species nor *L. monocytogenes* was confirmed in any of the boiled or pasteurized milk samples obtained from the market. This result is consistent with that of Moshoeshoe and Oliver (2012) and Sarker and Ahmed (2015) where no *Listeria* species were found in pasteurized milk. Kells and Gilmour (2004) found pasteurized milk to be free from *L. monocytogenes*, but occasionally isolated *L. welshimeri* which was later suggested occurring as a result of post processing contamination. However, a study conducted by Mouro *et al.* (1993) investigated the incidence of *Listeria* species and *L. monocytogenes* in 440 raw milk and pasteurized milk samples in Brazil and found that *Listeria* species was 0.9% in pasteurized milk whilst *L. monocytogenes* was not detected. Similarly, the study carried out by Ahrabi *et al.* (1998) showed an incidence of 5% *Listeria* and none *L. monocytogenes* in pasteurized milk. All these reports point to the fact that *L. monocytogenes* rarely survive pasteurization temperatures. However, Seyoum *et al.* (2015) recently reported a much higher rate of 40% contamination of *Listeria* species and 20% contamination of *L. monocytogenes* in pasteurized milk in Ethiopia. The presence of *Listeria*, particularly, *L. monocytogenes* in pasteurized milk is a great concern to public health since it may not undergo further processing before consumption. Improper pasteurization of milk and milk products and recontamination of pasteurized milk are possible reasons why the pathogen is found in pasteurized milk. Seyoum *et al.* (2015) indicated that, pasteurization of milk is not a guarantee for the safety of milk. After all, pasteurized milk has been implicated with an

outbreak of *L. monocytogenes* infection (Gellin *et al.,* 1991; Lyytikainen *et al.,* 2000). However, the findings in this study suggest that the pathogen *L. monocytogenes* was not detected in pasteurized milk. The possible explanations for the absence of the pathogen in market fresh milk are; adequate milk boiling/pasteurization and high maintenance of hygiene at sale point. This shows that consumption of pasteurized milk sold in the market may not pose a serious health threat to the public in the Northern region of Ghana.

Soft cheese samples had the highest prevalence of *Listeria* species and *L. monocytogenes* as determined by both the Enrichment PCR and Colony PCR techniques. The prevalence of *Listeria* species in the white soft cheese was 33.3%. Arslan and Ozdemir (2008) found 33.1% of 142 white cheese samples to be positive for *Listeria* species, while Shamloo *et al.* (2014) found all tested traditional cheese samples to be negative for *Listeria* species and *L. monocytogenes* respectively. Others reported lower prevalence than that reported in this study. Garedew *et al.* (2015) recovered 12.5% *Listeria* species from 40 cottage cheese samples and Alzubaidy *et al.* (2013) reported 20% incidence of *Listeria* species which are lower than that reported in this study. However, in a study by Seyoum *et al.* (2015) a much higher prevalence of 60% contamination of *Listeria* species in soft cheese has been reported in Ethiopia. In the same study they reported a rate of 26.7% contamination of *L. monocytogenes* which is much higher than the rate reported in this study. Pintado *et al.* (2005) earlier reported a much more prevalence of 75% *Listeria* species in soft cheese in Portugal. Seyoum *et al.* (2015) indicated higher levels of *Listeria* in cheese are as a result of either the production process or post- processing contamination. The soft cheese samples were obtained from producers and retailers. In Ghana, white soft cheeses are commonly produced and consumed by people. They were made by coagulating the milk with enzyme found in *Calotropis* procera. The enzyme present in the juice was added to coagulate the milk

whilst it was slowly heated on fire and once the desired coagulum was achieved, the cheese was further heated until it form curd. The cheese was then removed from the whey and moulded in to different sizes for sale. The cheese did not pass through ripening period. Though raw milk consumed by the Ghanaian populace is heat treated before consumption, it poses risk to the consuming population when it is processed into a product before pasteurization (Morobe *et al.,* 2009). The producers sell the cheeses to retailers who then process it for sale to the consumers. Retailers usually transport the cheese to selling point in ordinary water. The white soft cheeses are consumed as fresh product or as fried cheese. The latter is largely consumed by Ghanaians public. Cheeses sampled directly from producers and retailers both tested positive for *Listeria* indicating the in-efficiencies of the manufacturing process in eliminating the pathogen. The water content of the cheese, production process, pH and post- processing contamination are responsible for the presence of the organism in the cheese samples. Soft cheeses have higher water activity and thus allow greater growth of *Listeria* species (Meshref *et al.,* 2015). Insufficient hygiene, production environmental and the use of raw milk during the production of cheese could be responsible for the presence of *Listeria* in soft cheese. The survival of *Listeria* in cheese also depends on the pH of the cheese. Though the pH of the cheese samples in this study were not determined the pH of the 33.3% of the cheese samples that were tested positive lies within the range that promoted the growth of *Listeria*. *Listeria* species survive and grow in soft cheeses with a pH of 5.5 or above (Rogga *et al*., 2005).

Hence, it is possible that these *Listeria* positive cheese samples in this study had pH of 5.5 or above. The post-production handling of cheese involving the transfer of cheese into ordinary water and marketing chain could lead to contamination of cheese by *Listeria* species.

The presence of *Listeria* species and *L. monocytogenes* in white soft cheese poses public health risk although the fresh cheese may be further treated with sufficient heat by frying

before consumption. It is therefore important to state that those who patronize the fresh cheese are at risk of *Listeria* infections.

Yoghurts produced with starter culture investigated in this study were purchased from producers, market vendors and street sellers. In the present study the presence of *Listeria* species and *L. monocytogenes* were not detected in any of the experimental yoghurt samples obtained from the producers and street sellers. The percentage prevalence of both *Listeria* species and *L. monocytogenes* was found to be 0.0%. This result is consistent with that of Shamloo *et al.* (2014), where no *Listeria* species was detected in traditional yoghurt. Jayamanne and Samarajeewa (2001) and Rahimi *et al.* (2012) also reported the absence of *L. monocytogenes* in 30 and 55 samples of yoghurt respectively. However, studies conducted by other researchers disagree with the findings of this study. In Ethiopia, a total of 20 yoghurt samples were obtained from supermarkets and analyzed for the presence of *L. monocytogenes*, of which 1(5%) was found to be positive. In this same study *Listeria* prevalence in the yoghurt was found to be 10% (Seyoum *et al.*, 2015). Similar results found by Muhammed *et al.* (2013) where the prevalence of yoghurt contamination by *Listeria* species was 4% and *L. monocytonegenes* was 2%. In a related study in Uganda, Mugampoza *et al.* (2011) reported a much higher prevalence of *Listeria* species to be 30% and *L. monocytogenes* to be 3% in Locally Processed Yoghurt (LYP). The yoghurt samples examined in this study did not contain *Listeria.* In the Northern Region of Ghana, the milk is boiled heavily for at least 30 minutes prior to manufacture of the yoghurt. The high temperature treatment of milk is much more effective in destroying *Listeria.* Also, lactic acid bacteria form a major component of the yoghurt and this suppresses the growth of pathogens by producing bacteriocins (Jayamanne and Samarajeewa, 2001; Benkerrom *et al.*, 2003). The micro-flora of fermented milk products increased the death rate of *L. monocytogenes* when

storage is done at 20 °C (Stanczak *et al.*, 1997). Jayamanne and Samarajeewa (2001) indicated that conversion of milk in to yoghurt appear to be the most reliable means of controlling *L. monocytogenes* than pasteurization. In the Northern Region, the yoghurt is usually consumed by the public in frozen state. The frozen state of the yoghurt has nothing to do with the absence of *Listeria* in the yoghurt. Rahimi *et al.* (2010) showed that *L. monocytogenes* thrives at refrigeration temperature and that freezing has no lethal or sub-lethal effect on the survival of *L. monocytogenes*. Therefore, absence of *Listeria* in the yoghurt samples tested is probably due to less contamination of raw materials and partly to adequate application of good manufacturing practices. In fact, the manufacturing process ensures total elimination of the pathogen, especially if the raw materials were contaminated. The yoghurt sold to the public is packed in sterile plastic rubber and sealed by tying a knot at one end. This type of packaging used by the yoghurts producers reduces or eliminates post processing contamination and is a deviation from Danso-Boateng and Frimpong (2013) who indicated that the practice of hand tying of sachet water may serve as a potential source of bacteria contamination. The absence of *Listeria* in the yoghurt samples examined indicates that there is no health threat associated with consumption of the product.

The spontaneously fermented milk had prevalence of the *Listeria* species and *L. monocytogenes* to be 11.5% and 3.8% respectively. The results in this study is nearly in agreement with Mugampoza *et al.* (2011), who reported of 15% prevalence of *Listeria* species in traditionally fermented milk produced from unpasteurized milk. However, it is contrary to other similar studies. For instance, in a study by Mugampoza *et al.* (2011) *L. monocytogenes* was not in detected traditionally fermented milk. El Marnissi *et al.* (2013), however, reported higher incidence of 5.2% *L. monocytogenes* in *Lben* (traditionally fermented skimmed milk) produced in the city of Fez in the central north of Morocco. The

spontaneous fermented milk investigated in this study was purchased from producers, market vendors and street sellers. The spontaneous fermented milk is produced at a small scale by using natural fermentation. The milk used for the production was not pasteurized prior to production. Starter culture was not involved in the processing. The detection of *Listeria* species in spontaneous fermented milk could be attributed to several factors. For example, it may be due to heavy contamination of the raw milk used with *Listeria*. Additionally, it may be due to the fact that the rate of acid formation was slow (Mugampoza *et al.*, 2011). Also, it may be due to high pH of the yoghurt samples tested. The work conducted by Ahmed *et al.* (2014) where the number of inoculated *L. monocytogenes* counts in yoghurt samples decreased little when the acidity percentage was increased from 0.15- 0.83% and pH decreased from 6.63- 4.29 respectively, however as the acidity percentage reached 1.36% and pH reduces to as low as 3.86, the inoculated *L. monocytogenes* couldn't be detected in the yoghurt samples. This means that the slow rate of acid formation and the high pH of the heavily contaminated fermented milk could not prevent the survival of *Listeria*. However, Gahan *et al.* (1996) reported that *Listeria* species tolerate severe acid stress (pH 3.5) when they are induced by mild acid condition (pH 5.5); this phenomenon is termed acid tolerance response. Barker and Park (2001) also indicated that the most acid tolerant food-borne pathogen is *L. monocytogenes.* The hygienic conditions regarding milking, handling and storage are other factors that contribute to contamination of fermented milk products by *Listeria* (Dalu and Feresu, 1996). It is however important to state that the detection rate of *L. monocytogenes* was very low. The reason for the low isolation rate of *L. monocytogenes* could be attributed to low pH (3.5-4.0) of the final product and the production of other organic acids and bacteriocins. Additionally, competition between *L. monocytogenes* and natural flora of the spontaneous fermented milk on the growth or nutritional requirement could hinder the growth of *L. monocytogenes* (Zamani-Zadeh, 2011). Bess *et al.* (2005)

indicated that the natural flora of contaminated foods has no effect on *Listeria* species in each enrichment steps and that the final changes of species are due to the nutritional competitions. In conclusion one can state emphatically that, the results of this study demonstrate unhygienic handling and storage, and we recommend improvement of the hygiene. The results of this study show that there is a risk of *Listeria* infection for consumers of spontaneously fermented milk sold in the Northern Region of Ghana.

5.4 Sensitivity of colony PCR and Enrichment PCR in detecting *Listeria* and *L. monocytogenes*

In this study, the colony PCR detected 21 *Listeria* contaminated samples and that of Enrichment PCR detected 22 *Listeria* contaminated samples suggesting that the Enrichment PCR is sensitive in detecting *Listeria* species than colony PCR. The result of this study is in agreement with that of Lakićević *et al.* (2010) who detected 17 *Listeria* contaminated samples directly from pre-enrichment (24h) broth using PCR and 10 *Listeria* contaminated samples using cultured method with confirmation by PCR. AL-Ashmawy *et al*. (2014) also reported similar findings, but in their study only *L. monocytogenes* was detected. Interestingly, out of the 163 samples examined, 11 were found to be positive for *L. monocytogenes* by both methods. This finding is in agreement with Jeršek *et al.* (2005) who found 8 out of 31 natural contaminated food samples to be positive for *L. monocytogenes* by using both cultural and PCR-based methods and Holko *et al*. (2002) who detected *L. monocytogenes* in 18 out of 100 milk and dairy products by using both the cultural and nested PCR methods.

The colony PCR gave the ready results after 9 days of culturing and incubation on selective media. In this study longer period of incubation does not produce better results as compared to the results obtained by Holko *et al.* (2002). Manzano *et al.* (1997) indicated that the conventional culture method is laborious and time consuming, and needs about 5 to 10 days

to complete. This affects the food industries in the area of export or import by slowing down the distribution process. To overcome this challenge, the PCR detection of pathogenic organisms from 24 h or 48 h enriched broth was introduced (Holko *et al.*, 2002).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. The dairy farm owners who produce milk and the processors of dairy products have the awareness that the milk and milk products can get contaminated if special hygienic practices are not adhered to.

   The prevalence of *Listeria* species in soft cheese, raw milk and spontaneously fermented yoghurt were 33.3%, 17.5% and 11.5% respectively whiles pasteurized milk, fried cheese and yoghurt produce with starter culture were not contaminated with *Listeria.*

2. *L. monocytogenes* was detected in 18.5%, 8.8% and 4% of soft cheese, raw milk and spontaneously fermented milk samples respectively.
3. Raw milk, Soft cheeses and spontaneously fermented milk sold in the Northern Region of Ghana pose a risk of *Listeria* infection to consumers. However, the fried cheese, pasteurized milk and yoghurt produced with starter culture do not pose any risk of *Listeria* infection to consumers.
4. There was no significant difference in the performance of the two agar media used in this study.
5. The enrichment PCR technique and colony PCR techniques were not significantly different in their ability to detect *L. monocytogenes*.

6.2 Recommendations

1. Producers of fresh milk at the dairy farms should ensure that the udders and teats of milking cows are properly washed or disinfected before the commencement of milking. This will reduce the level of contamination of fresh milk from dairy farms.
2. Producers, processors and vendors of milk and its products should observe high level of personal hygiene and sanitary practices whiles handling milk
3. Pasteurized milk should be used to manufacture soft cheese and spontaneous fermented milk to minimize the risk of *Listeria* infection.
4. Risk assessment should be applied at various levels of the dairy value chain which show the current state of knowledge about contamination of milk by *L. monocytogenes*.
5. Further studies should be directed at identifying the specific sources of *Listeria* species in the processing environments. This will help in controlling the incidence of these organisms in fresh milk and processed milk.

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APPENDICES

Appendix 1: Questionnaire on value chain analysis of the dairy sector in Northern region, Ghana.

UNIVERSITY FOR DEVELOPMENT STUDIES

BIOTECHNOLOGY DEPARTMENT

A VALUE CHAIN ANALYSIS OF THE DAIRY SECTOR IN THE NORTHERN REGION, GHANA

Consent: I wish to seek your consent and support to carry out a study on the above subject. I wish to assure you that every information provided here will be used mainly for academic purposes and where necessary to advocate for policy intervention.

Part a: Demographic profile

1. Sex

   Male [ ]

   Female [ ]

2. Age

   ≤ 20 [ ]

   21 – 30 [ ]

   31 – 40 [ ]

   41 – 50 [ ]

   51 – 60 [ ]

   ≥ 61 [ ]

3. Education

   None [ ]

Basic [ ]

Secondary/Technical/Vocational [ ]

Tertiary [ ]

Part b: Milk Production

4. Which of the following procedure do you use in milking?

a. Machine [ ]

b. Hand with gloves [ ]

c. Hands without gloves [ ]

5. Which of the following tools do you use in milking? Choose as many as possible.

a. Calabash bowl [ ]

b. Rubber container [ ]

c. Metal bowl [ ]

6. How many cattle do you milk per day?

a. 1-5 [ ]

b. 6-10 [ ]

c. 11-15 [ ]

d. 16+ [ ]

7. How many times do you milk per day? If b or c move to Q5 else Q6

a. Once [ ]

b. Twice [ ]

c. Thrice [ ]

8. Do you wash milk containers after the first milking in a day?

a. Yes [ ]

b. No [ ]

9. If yes to Q5, do you wash using soap and water?

a. Yes [ ]

b. No [ ]

10. How many times do you wash milk containers in a day?

a. Once after milking [ ]

b. Once before milking [ ]

c. Twice (before and after) [ ]

11. Do you wash/disinfect your hands before milking?

a. Yes [ ]

b. No [ ]

12. Do you usually disinfects the udders of your cows before milking?

a. Yes [ ]

b. No [ ]

13. Do you milk with wet or dry containers?

a. Wet container [ ]

b. Dry container [ ]

14. What is the source of water used?

a. Pipe water [ ]

b. Hand dug wells [ ]

c. Dam [ ]

d. River/lake/streams [ ]

e. Other specify ……..

15. What quantity of milk do you produce per day?

a. 1-10 litres [ ]

b. 11-20 litres [ ]

c. 21-30 litres [ ]

d. 31-40 litres [ ]

e. 40+ litres [ ]

16. From how many cows do you obtain this quantity of milk?

a. 1-5 [ ]

b. 6-10 [ ]

c. 11-15 [ ]

d. 16-20 [ ]

e. 20+ [ ]

Part c: Management practice

17. How do you house your cattle?

a. Fence [ ]

b. Room [ ]

c. Open space [ ]

d. Tethering [ ]

e. Other specify…….

18. How do you feed your cattle?

a. Free range [ ]

c. Silage [ ]

d. Other specify ……..

19. Do you have any linkage with veterinary officers?

a. Yes [ ]

b. No [ ]

20. If yes, how often do they visit your dairy farm?

a. Regularly [ ]

b. Only when the cattle are sick [ ]

c. Both vaccination and treatment [ ]

21. Do you often observe any clinical signs of illnesses among your cattle?

a. Yes [ ]

b. No [ ]

22. If yes, which of the clinical signs do you observe among the cattle? Choose as many as possible

a. Conjunctivitis/pinkeyes [ ]

b. Weakness [ ]

c. Mastitis [ ]

d. Diarrhoea [ ]

e. Abortion [ ]

f. Others, Specify [ ]

23. Which of the following diseases attack your cattle? Choose as many as possible

a. Tuberculosis [ ]

b. Brucellosis [ ]

c. Anthrax [ ]

d. Cutagneous bovine pleura pneumonia [ ]

e. Haemorrhagic Scepticemia [ ]

f. Salmonellosis [ ]

g. Black quarter [ ]

f. Others, Specify [ ]

Part d: Sale of milk

24. Do you sieve the milk before selling?

a. Yes [ ]

b. No [ ]

25. Do you boil the milk before selling?

a. Yes [ ]

b. No [ ]

26. If yes to Q20 and Q21, why? choose as many as possible

a. To remove dirt/germs [ ]

b. To keep it long [ ]

c. Other specify ……

27. Do you sell your milk or you turn it in to a milk product before selling?

a. Milk [ ]

b. Milk product [ ]

c. Both [ ]

28. Which of the following products do you turn your milk in to? Choose as many as possible

a. Soft Cheese [ ]

b. Spontaneously fermented yoghurt [ ]

c. Yoghurt with starter culture [ ]

d. Milk powder [ ]

e. Milk butter [ ]

f. Other specify …….

29. If answer to Q28 is (a), then tell me the production process

……

……

……

……

……

30. If answer to Q28 is (b), tell me the production process

……

……

……

…

31. If answer to Q28 is (c), tell me the production process

……

……

……

……

……..

32. If answer to Q28 is (d), tell me production process

……

……

……

……

..

33. If answer to Q28 is (e), tell me the production process

……

……

……

……

……

34. Do you sell the milk to consumers yourself or you sell to retailers?

a. Consumers [ ]

b. Retailers [ ]

c. Both [ ]

35. How do you transport your milk to consumers for sale?

a. Carrying on head [ ]

b. Market trucks [ ]

c. Cycles [ ]

d. Other specify ……..

36. How long does it take to get to the selling point?

a. Less than 30 minutes [ ]

b. 30-60 minutes [ ]

c. 60+ minutes [ ]

37. Do you wear gloves or wash your hands before selling milk to the consumer?

a. Wear gloves [ ]

b. Wash hands [ ]

c. None of the above [ ]

38. Where do you sell your milk to consumers?

a. Sit at a sale point to sell [ ]

b. Move round to sell [ ]

c. Other specify……

Thank you for your cooperation

Appendix 2: Culture Media used

Chemical composition of Buffered peptone water (Oxoid CM0509)

Composition	gm/litre
Peptone	10.0
Sodium chloride	5.0
Disodium phosphate	3.5
Potassium dihydrogen phosphate	1.5
pH 7.2 ± 0.2	

Chemical composition of *Listeria* Enrichment Broth Base (Oxoid, CM0862)

Composition	gm/litre
Tryptone soya broth	30.0
Yeast extract	6.0
pH 7.3 ± 0.2	

Chemical composition of *Listeria* Selective Enrichment Supplement (Oxoid, SR0141)

Vial contents	per vial	per litre
Nalidixic acid	20.0 mg	40.0 mg
Cycloheximide	25.0 mg	50.0 mg
Acriflavine hydrochloride	7.5 mg	15.0 mg

Chemical composition of PALCAM Agar Base (Oxoid, CM0877)

Composition	gm/litre
Columbia Blood Agar Base	39.0
Yeast extract	3.0
Glucose	0.5
Aesculin	0.8
Ferric ammonium citrate	0.5
Mannitol	10.0
Phenol red	0.08
Lithium chloride	15.0

pH 7.2 ± 0.2

Chemical composition of PALCAM Selective Supplement (Oxoid, SR0150)

Vial contents	per vial	per litre
Polymyxin B	5.0 mg	10.0 mg
Acriflavine hydrochloride	2.5 mg	5.0 mg
Ceftazidime	10.0 mg	20.0 mg

Chemical composition of *Listeria* selective agar (Oxford formulation) (CM0856)

Composition	gm/litre
Columbia Blood Agar Base	39.0
Aesculin	1.0
Ferric ammonium citrate	0.5
Lithium chloride	15.0

pH 7.0 ± 0.2

Chemical composition of *Listeria* Selective Supplement (Oxford formulation, SR0140E)

Vial contents	per vial	per litre
Cycloheximide	200 mg	400 mg
Colistin sulphate	10.0 mg	20.0 mg
Acriflavine	2.5 mg	5.0 mg
Cefotetan	1.0 mg	2.0 mg
Fosfomycin	5.0 mg	10.0 mg

Chemical composition of Tryptone Soya Agar (Oxoid, CM0131)

Composition	gm/litre
Tryptone	15.0
Soya peptone	5.0
Sodium chloride	5.0
Agar	15.0

pH 7.3 ± 0.2

Appendix 3: Detection protocol

Weigh 25g or ml of dairy sample and add to 225 ml of 1% BFW

↓

Homogenize milk products in a blender

↓

Incubate homogenate at 30 °C for 24 h

↓

Add 10 ml of homogenate to 90 ml Listeria enrichment broth base with selective enrichment supplement

↓

Incubate for 48 h at 30 °C

↓

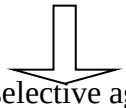
Subculture broth in selective agar plates (Oxford and Palcam respectively)

↓

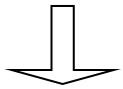
Incubate for 48 h at 37 °C

↓

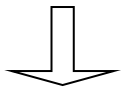
Observe for Grey-green colonies surrounded by black zones for oxford plates or grey colonies and black halo against a cherry-red background for palcam plates



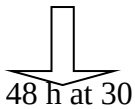
Select at least 2 inferred *Listeria* colonies on selective agars and purify these on tryptone soya agar



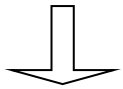
Subject isolates to gram's and catalase reactions



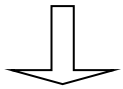
Grow colonies that are both gram positive and catalase positive on Oxford and palcam agars



Incubate for 48 h at 30 °C



Extract DNA by thermal lysis method



Subject DNA to PCR and electrophoresis

Photographed

Appendix 4: Statistical Analysis

Table 1: Rates of performance of oxford and palcam agars in detecting *Listeria*

PALCAM	20	18	38	21.639	16.361
OXFORD	21	13	34	19.361	14.639
	41	31	72		

P =0.43

Table 2: Relationship between the true/false positive results and the food categories

Palcam	123	2	125	123.524	1.476
Oxford	128	1	129	127.476	1.524
	251	3	254		

P=0.54

Table 3: Comparing Enrichment and Colony PCR

Colony PCR	21	142	163	7.693	49.31
Enrichment PCR	22	141	163	14.307	91.693
	43	283	326		

P=0.27

Appendix 5: Unhygienic Milking and handling of milk that can contaminate milk with *Listeria*

Appendix 6: Steps in soft cheese processing