Incidence of *Listeria monocytogenes* in milk from producers in the Maseru area

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By

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DECLARATION

I, the undersigned, declare that this dissertation is submitted to the Central University of Technology, Bloemfontein in fulfillment for the degree of MAGISTER TECHNOLOGIAE: Biomedical Technology in the Faculty of Health and Environment Sciences, School of Health Technology. The work contained herein is my original work with exemption to the citations and that this work has not been submitted at any other University in partial or entirety for the award of any degree.

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ABSTRACT

The objective of this study was to determine the prevalence of *Listeria monocytogenes* and also to assess the general hygiene of fresh milk in the Maseru area, Lesotho. A total of 200 milk samples (40 pasteurised and 160 raw milk samples) were used for the research. Raw milk samples were collected from the local farmers at the Dairy reception as they bring it for selling. Pasteurised milk samples were bought from different milk selling points in the Maseru area. The total aerobic plate count, total coliform count and total *E. coli* count for 160 raw milk samples and 40 pasteurised samples were performed to determine the quality of milk.

Milk was enriched in selective broths to increase detection sensitivity and was directly plated on selective agars for direct bacterial enumeration. About 54.4% of the of the raw milk samples had total aerobic plate counts greater that 200 000 cfu/ml while 55.6% (89/160) of the raw samples had high counts of greater than 20 cfu/ml for total coliforms, and 21.9% (35/160) of the samples had higher than expected total *E. coli* counts. High total coliform count was detected in 17.5% (7/40) of the pasteurised milk samples and about 67.5% (27/40) of these samples exceeded the limit for total aerobic plate counts. The counts exceeded the milk standards for pasteurised milk. Phosphatase activity was detected in seven pasteurised milk samples, whereas 33 tested negative for phosphatase activity. Some pasteurised milk samples tested positive for coliform counts which exceeded the maximum limits according to national standards for pasteurised milk. However, most of the pasteurised samples (82.5%) had acceptable counts of less than 20 cfu/ml. API and PCR were used for confirmation and amplification of the isolated *Listeria* strains. The prevalence of *Listeria* was found to be (3.75%). *Listeria* species were found in 6 out of 200 samples tested (160 raw milk samples and 40 pasteurised milk), and were

only detected in the raw milk samples. Five species belonged to *Listeria monocytogenes* and one was *Listeria innocua*. None of the *Listeria* was detected in the pasteurised milk samples. Serotyping was done through multiplex PCR with D1, D2, FlaA and GLT primers to determine the serovar groups of *L. monocytogenes*. All six isolates revealed 214 bp gene which identifies the serotypes in Lineages I or III. The genetic fingerprinting of the isolated *Listeria* was also determined. Enterobacterial Repetitive Intergenic Consensus (ERIC) sequence-based PCR was used to generate DNA fingerprints with ERIC specific primers. On the basis of ERIC-PCR fingerprints, three different DNA patterns could be discriminated among the analysed isolates. Three *L. monocytogenes* isolates showed similar DNA banding patterns, while two isolates both had different profiles. A questionnaire was used to determine consumption of raw (unpasteurised) milk or pasteurised milk and its products and it was completed by 300 households from the community. Although there was no indicated prevalence of raw (unpasteurised) milk consumption from the community, participants indicated symptoms alleged to consumption of pasteurised milk and/or milk products. According to community perception some of the dairy products consumed were allegedly implicated in food poisoning illnesses experienced. Participants indicated more symptoms with both fresh and sour milk consumption than in cheese and yogurt consumption.

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LIST OF ABBREVIATIONS

API	Analytical profile index
Bp	Bacterial phosphatase
CDC	Center for Disease Control
cds	Coding sequence
CFSAN	Center for Food Safety and Applied Nutrition
cfu	Colony forming units
DNA	Deoxyribonucleic acid
DoH	Department of Health
ERIC	Enterobacterial Repetitive Intergenic Consensus
FAO/WHO	Food and Agricultural Organization/ World Health Organisation
FDA /CFSAN	Food and Drug Administration/Center for Food Safety and Applied Nutrition
FSIS/USDA	Food Safety and Inspection Service/ United States Department of Agriculture
GPC	Gram positive cocci
GPCB	Gram positive cocco-bacilli
HACCP	Hazard Analysis Critical Control Point
ICMSF	International Commission on Microbiological Specifications for Foods
IDF	International Dairy Federation
IFT	Institute of Food Technologists

IGEM	International Genetically Engineered Machine competition
LM	Low melting
L. monocytogenes	*Listeria monocytogenes*
L. innocua	*Listeria innocua*
L. ivanovii	*Listeria ivanovii*
L. welshimeri	*Listeria welshimeri*
L. murrayi	*Listeria murrayi*
L. seeligeri	*Listeria seeligeri*
L. grayi	*Listeria grayi*
MCC	Maseru City Constituencies
NCBI	National Center for Biotechnology Information
Neg	Negative
Pos	Positive
PCR	Polymerase Chain Reaction
REP	Repetitive Extragenic Palindromic
rpm	rotation per minute

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CHAPTER I

LITERATURE REVIEW

1.1 INTRODUCTION

Food-borne illness is a serious public health concern as it affects many people and contributes to the mortality rate. As far back as 1999 the United States of America (USA) Centre for Disease Control (CDC) estimated that annually 76 million people in the USA contract some form of food poisoning of which more than 300 000 are hospitalised and 5 000 die (Mead *et al.* 1999). In 2007, food-borne disease outbreaks were reported to the CDC and as a result of these outbreaks 21 244 illnesses and 18 deaths were reported by state investigators (MedNews, 2010). In a survey done by Scallan *et al.* (2011) it was estimated that in the USA 9.4 million episodes of food-borne illnesses reported were caused by 31 major pathogens. In South Africa statistics from 2006 reported that 1 886 food-borne illnesses were recorded between 2001 and 2005 and 51 fatalities were reported (South Africa, Department of Health, 2006). In South Africa such low incidences are probably the result of most cases not being reported.

Food manufacturers and healthcare professionals have a responsibility in the prevention and control of food-related diseases especially during this period when public interest and concern for food safety are high due to the increase of immunocompromised people in communities. The increasing incidence of HIV/AIDS in the community can be regarded as one of the factors contributing to the susceptibility of individuals to food-borne illnesses (Mead *et al.,* 1999; Tan *et al.*, 2004). In healthy individuals, food-borne infections do not always produce disease due to the body's strong defence mechanisms, but in immunocompromised individuals defence mechanisms are usually overwhelmed and

minor infections often result in illness (Cliver, 1990). People primarily at risk of contracting food-borne illness are the very young, the elderly and also immunocompromised individuals (Johnson *et al.,* 2004; Wojciech *et al.,* 2004; Kirkan *et al*., 2006; Stepanovic *et al.,* 2007).

Most food-borne diseases have been contracted through ingestion of food of compromised quality, which is often contaminated with pathogenic microorganisms or their toxins (Gerner-Smidt & Whichard, 2007). Pathogenic bacteria such as *Shigella* spp., *Listeria monocytogenes* and *Yersinia enterocolitica* have been reported in several cases as the causative agents of food-borne infections (Jemmi & Stephan, 2006; Hamdi *et al*., 2007). These organisms are found in contaminated food (Gouws & Liedemann, 2005) and are able to survive and proliferate in foods like milk and milk products (Hayes *et al.,* 1986; Holko *et al*., 2002; Millet *et al.,* 2006; De Santis *et al.,* 2007) and other food products (Vaz-Velho *et al.,* 2001). Although cold storage is used as a form of preservation, pathogens such as *L. monocytogenes* grow well at 4°C (Champagne *et al*., 1994; Rodriguez-Lazaro *et al*., 2004; Ryser & Marth, 2007) and have been implicated in cases of food-borne illnesses and outbreaks (Lunden *et al*., 2004; CDC, 2009) with a mortality rate of about 28% (Institute of Food Technologists, 2004).

1.2 MILK AS A POTENTIAL SOURCE OF INFECTION

Milk is a natural source of nutrition for infant mammals but is also used as a food product for humans of all ages. As such it is important that it is provided free from disease causing agents. Even though milk and milk products provide nutrition benefits, raw milk, in particular, can harbour dangerous microorganisms that can pose serious health risks to the consumer. Raw milk refers to any milk that has not been pasteurised and has the

potential to contain pathogens that are often associated with food-borne illnesses (Mahanta, 1984; Hobbs & Roberts, 1993; Arakawa *et al*., 2008). Food-borne infections and intoxications in humans have resulted from the consumption of contaminated foods, of which meat and dairy products are most frequently implicated due to their high spoilage potential (Rowe, 2003; Arakawa *et al.,* 2008).

1.2.1 BACTERIAL PREVALENCE IN MILK

The natural composition of milk makes it an excellent source for microorganisms to grow. It is, therefore, important to maintain high pre-processing standards to avoid contamination (Van Kessel *et al.*, 2004) because both pre-processing and post-processing measures are crucial in maintaining low numbers of acceptable standards (Goulet *et al.*, 2001; Lukinmaa *et al.*, 2003). Legislature stipulates the minimum numbers permitted for microbial content in both raw and pasteurised milk. Milk should, therefore, be tested for the presence of organisms, to ensure that counts are within the ranges as stipulated by Regulation 1555 of 21 November 1997 (South Africa, 1997). Refer to Table 1.1 for standard count values for milk.

Table 1.1 Standard bacterial count values for milk (Foodstuffs, Cosmetics and Disinfectants Act. (Act No.54 of 1972). Regulation 1555 of 21 November 1997).

State Of Milk	**Total Aerobic Count**	**Total Coliforms** (Dry Film Method)	**Total *E. coli***
Raw milk for processing	≤ 200 000 cfu/ml	≤ 20 cfu/ml	<10 cfu/ml
Pasteurised milk	≤ 50 000 cfu/ml	≤ 20 cfu/ml	Absent in 1ml

Milk is vulnerable to spoilage and must be kept refrigerated because if left standing without refrigeration, the lactic acid bacteria naturally ferment it to produce lactic acid, which gives raw milk its sour taste (Jay, 1992). The lactic acid bacteria convert lactose to lactic acid that acts as a natural preservative. Prolonged fermentation however, renders the milk unpleasant to consume as a result of the presence of coliforms in raw milk. These organisms may continue to proliferate and eventually produce an unfavourable odour and taste in milk (Mahanta, 1984). Pasteurisation is one of the methods employed to kill the pathogenic bacteria and to control milk quality. This is done by exposing raw milk to a high temperature of 72°C for 15 seconds and then cooling to below 10°C within 30 minutes (Jay, 1992). Although pasteurisation of raw milk will initially destroy any potential pathogens and increase its shelf-life (Jay, 1992), the presence of heat resistant organisms such as streptococci will eventually cause milk spoilage and render it unsuitable for consumption. This process may also occur in raw milk because of the presence of spoilage bacteria (Jay, 1992; Rowe, 2003).

Elimination of bacterial contamination is an important factor in the production of good quality milk (Allessandria *et al*., 2010). Milk production with low bacterial counts can be achieved through good farm management where aspects contributing to hygiene are taken into consideration to ensure the production of a quality product. Factors to be considered in maintaining hygiene include herd and milking parlour hygiene, milking practices, equipment handling and cold storage on farms. At the factory factors to be addressed include personal hygiene, handling of raw products, plant cleaning systems as well as the design of the plant. Raw milk generally contains varying numbers of microorganisms. Bacterial counts above the recommended range often relate to insufficient sanitation and hygiene practices depending on the care and handling of herds

and milk (Kelly *et al.,* 2009), such as less efficient cleaning of the milking system and unwashed udders and teats (Blowey & Edmondson, 2010). Failure in hygiene practises often results in contaminants that may affect the quality of the product. For example, coliforms are used as hygiene indicators and at the same time can act as post pasteurisation contaminants, which can compromise the quality of the product because apart from being pathogenic and their ability to produce toxins, the majority of these organisms are responsible for severe spoilage in dairy products (Arakawa *et al.,* 2008). It is evident that sources of contamination can be either animals or humans, in addition, soil and silage can be the source of pathogenic bacteria such as *L. monocytogenes, Pseudomonas aeruginosa* and *Clostridium perfringens* (Van Kessel *et al*., 2004). Shedding of organisms from animals suffering from mastitis has also been mentioned as a source of contamination and organisms such as *Salmonella* and *L. monocytogenes* have been implicated (Ryser & Marth, 1991; Van Kessel *et al*., 2004). Moreover, according to Blowey & Edmondson (2010), *E. coli* is one of the common organisms found in mastitic animals.

The problem this situation poses at a dairy production plant is that raw milk contaminated with zoonotic pathogens may provide a reservoir for recontamination of the final milk product at the processing plants. Although readily destroyed during pasteurisation, some bacteria still appear in pasteurised milk at low levels as post pasteurisation contaminants and food-borne illnesses have been reported where *L. monocytogenes* in pasteurised milk was implicated (Ryser & Marth, 2007; CDC, 2008). Other organisms which have been implicated in milk-borne infection include *Salmonella, Campylobacter, Yersinia enterocolitica, E. coli, Enterococcus faecalis, Streptococcus* and *Staphylococcus* (Eley, 1992; Bell & Kyriakides, 1998). Apart from the fact that some of these organisms may be

post pasteurisation contaminants, organisms such as *Micrococcus, Enterococcus, Lactobacillus, Streptococcus*, and *Corynebacterium spp*. are thermoduric bacteria that can survive pasteurisation (Arakawa *et al*., 2008).

1.2.2.1 GRAM POSITIVE ORGANISMS

According to a survey done by Kerr *et al.* (1993) *L. monocytogenes* is one of the pathogenic organisms often found in the dairy environment. Other Gram positive pathogens commonly found in milk and also in the environment include *Staphylococcus aureus*, enterococci and streptococci. Some of these organisms influence most of the total milk count (Jeffrey & Wilson, 1987; Bramley & McKinnon, 1990). Apart from the fact that the *S. aureus* may be shed from cows with mastitis, the organism is also very common on the hands and skin of man, and on cattle (Harding, 1995; IDF, 1996). Enterococci usually act as contaminants of meat and milk either as environmental contaminants (Giraffa, 2002) or as post processing contaminants (Hugas *et al.*, 2003). However, *Enterococcus faecium* has been found to produce bacteriocins that inhibit *L. monocytogenes* growth, thereby proving to be beneficial if present in milk (Chanos & Williams, 2011).

1.2.2.2 GRAM NEGATIVE ORGANISMS

Gram negative organisms found to be responsible for milk contamination are *Klebsiella pneumonia*, *E. coli*, *Citrobacter* spp, *Serratia* spp and *Enterobacter aerogenes* (Bramley & McKinnon, 1990). Since *E. coli* is always present in faeces (natural in the colon of animals), it is often used as an indication of faecal contamination. Contamination of food with faecal coliforms usually serves as an indication of unsanitary production practices in milk production as well as the potential presence of pathogenic organisms (Van Kessel *et al*., 2004). *Yersinia enterolitica* is another bacterium that has been found in milk in large

quantities. In a study done in the United Kingdom, this organism was isolated from approximately 50% of raw milk samples. Since outbreaks have been traced to pasteurised milk, controversy has arisen over its resistance to heat as it has been isolated in ultra-high temperature treatment (UHT) milk (Eley, 1992).

1.2.3 MILK AND/OR MILK PRODUCT CONSUMPTION AND CULTURAL BELIEFS IN THE COMMUNITY

In rural Lesotho communities it is common practice to rear cattle for milk provision for personal use while the excess milk is sold to supplement the household income. Communities in rural areas often do not have access to cold storage facilities in their homes and consequently milk is often kept at room temperature. This creates an opportunity for the spoilage bacteria to multiply and eventually render the milk unsuitable for consumption. Sour milk that is traditionally prepared from raw milk is preferred by most consumers, as there is a belief that it tastes better than commercially prepared sour milk and it can be kept in the household for longer periods than fresh milk (anonymous). Others believe that consumption of pasteurised milk can make people sick. However, both raw milk and pasteurised milk have a potential of causing illness, while at the same time milk can cause allergic reactions in people sensitive to milk proteins or people who are lactose intolerant (Altug, 2003). Moreover, there is no meaningful difference in the nutritional values of pasteurised and raw milk except for the fact that pasteurised milk contains lower levels of bacteria that can cause food spoilage (Eley, 1992).

1.3 LISTERIA

Listeria is a recognised human pathogen, with the most pathogenic strain being *L. monocytogenes* while *L. ivanovii* is pathogenic to animals (Roberts & Wiedmann, 2003; Brugère-Picoux, 2008). *Listeria* is widely distributed and can be found almost everywhere in the environment. As a result of its wide distribution among animals and in the environment and its ability to survive and grow at low temperatures, *Listeria* has become a challenge to the food industry. Listeriosis is a clinically significant disease because of its mortality and severity, and food-borne outbreaks associated with this organism have been reported mainly due to consumption of raw milk and/or raw milk products. Outbreaks associated with pasteurised milk usually occur due to improper pasteurisation or post pasteurisation contamination (Van Kessel *et al*., 2004; Ryser & Marth, 2007). Raw milk or foods made from raw milk may contain the bacteria, making it difficult to control especially within the food environment. Conventional and molecular methods have been employed over decades to isolate *Listeria* in food, clinical and environmental samples (Ryser & Marth, 2007; Yi Chen & Knabel, 2007).

Listeria species are found in living and non-living matter, mainly soil, vegetation, silage, sewage, humans, animals and water (Ryser & Marth, 1991; Eley, 1992; Bubert *et al*., 1999; Dimaio, 2000; Rodriguez-Lazaro *et al*., 2004; Kirkan *et al*., 2006; Liu, 2006). *Listeria* species show resistance to environmental stress and are resistant to concentration of salt, refrigeration and low pH (Bubert *et al*., 1999; Becker *et al*., 2000; Beales, 2003; Rodriguez-Lazaro *et al*., 2004; Conter *et al*., 2006: Liu, 2006; Gandhi & Chikindas, 2007; Najjar *et al.*, 2007). *Listeria* is capable of growth in 10% NaCl (Robinson *et al*., 2000). Nisin is a natural preservative used in cheese and yoghurt and has shown an inhibitory effect on the growth of *Listeria* (Gandhi & Chikindas, 2007). However, resistance to nisin

has been reported (Collins *et al*., 2011). Acidity is a primary factor in preservation of fermented food (Heavin *et al*., 2009), but *Listeria* is able to grow at a pH of 5.0 (Robinson *et al*., 2000) and cheese with a pH range of 4.9 to 7.7 have shown an ability to support the growth of *Listeria*. However, cheese with a lower pH showed inhibition of listerial growth (Hui *et al*., 1994). De Azerêdo *et al*. (2012) has recently identified essential oils from herbs that demonstrated inhibitory effect against *L. monocytogenes* growth in a study by Mahmoud *et al*. (2012), X-ray treatment of *L. monocytogenes* has shown to be effective in eliminating the organism in refrigerated catfish. *Listeria* can grow over a wide range of temperatures ranging from 2°C to 50°C (Rodriguez-Lazaro *et al*., 2004; Ryser & Marth, 2007). Storage temperature of around 4°C is not sufficient to control proliferation of *L. monocytogenes* as even small bacterial inoculums held at these temperatures may outgrow competing organisms and produce an infective dose sufficient to cause disease (Eley, 1992; Ryser & Marth, 2007). The organism can also survive drying and freezing and is regarded as less sensitive to heat treatment than *Salmonella* and *Campylobacter* (Eley, 1992). Some authors debate its capability to survive pasteurisation (Hui *et al*., 1994; Vardar-Unlu *et al*., 1998; Ryser & Marth, 2007) and other studies suggest that acid shock and cold shock may lead to cross-protection to heat or high pressure treatments (Beales, 2003; Gandhi & Chikindas, 2007*).* In one study, it was found that *L. monocytogenes* failed to be inactivated when present in large numbers in samples subjected to the high-temperature short-time (HTST) pasteurisation. On the contrary, most literature still confirms its sensitivity to pasteurisation temperature (Jay, 1992; Lunden *et al.,* 2004), and as thus it is still considered an effective method. Friedly *et al.* (2008) observed that survival times for *Listeria* were reduced when exposed to temperatures between 62.5°C and 70°C. It was also observed that it is able to survive relatively high osmotic pressures (Eley, 1992). Considine *et al.* (2011) confirmed that a proline synthesis system present in

L. monocytogenes confers survival to high pressure treatments. Other authors suggest that various preservation conditions like refrigeration, extended shelf life and lack of oxygen, may permit resuscitation and growth of injured cells (Mendonca *&* Knabel, 1994; Vardar-Unlu *et al*., 1998; Ryser & Marth, 2007).

Within the food and dairy industry *Listeria* has been isolated from biofilms (Vilar *et al*., 2007; Simoes *et al.,* 2010). Alessandria *et al.* (2011) conducted a study on contamination in a dairy plant and observed a high capability of *L. monocytogenes* to attach to abiotic surfaces. This was also observed by Holah *et al.* (2002) who isolated *Listeria* from biofilms where they had adhered to and colonised moist areas and equipment in a dairy plant. Bacteria can multiply to high numbers on biofilms and ultimately contaminate the finished products (Husu, 1990; Hood & Zottola, 1995). *Listeria* has been repeatedly isolated from the gaskets of a milk pasteurisation line and equipment (Carpentier & Chassaing, 2004; Carpentier & Olivier-Cerf, 2011; Doijad *et al*., 2011). *Listeria* has also been isolated from cleaned and disinfected areas (Simoes *et al*., 2010). This is not surprising as resistance to disinfectants has been reported (Bisbiroulas *et al*., 2011) making it difficult to control *L. monocytogenes* in plant equipment. Nilson *et al*. (2011) conducted a study to determine the extent to which *L. monocytogenes* biofilm production protects against a quaternary ammonium compound (QAC) disinfectant and increased QAC resistance in mature biofilms was reported.

1.3.1 *LISTERIA* SPECIES

Among the nine species of *Listeria*; *L. monocytogenes, L. welshimeri, L. seeligeri, L. innocua, L. ivanovii, L. murrayi, and L. grayi* (Ryser & Marth, 2007)*, L. rocourtiae* (Leclercq *et al*., 2010) and *L. marthii* (Graves *et al.,* 2010), only one species,

L. monocytogenes, is regarded as causing frequent illness in humans (Lopez *et al*., 2006). Only a few isolated cases of human infection caused by *L. ivanovii* and *L. seeligeri* (Eley, 1992; Guillet *et al.*, 2010; Lortholary & Lecuit, 2010), *L. grayi* (Todeschini *et al.*, 1998; Salimnia *et al*., 2010) and *L. murrayi* (Johnson *et al*., 2004) have been documented and these organisms are, therefore regarded as rare causes of illness in humans. *L. innocua* has been previously reported as non-pathogenic and there has not been any illness reported due to this organism until in 2003 when a fatal case of bacteraemia was reported by Perrin *et al.* (2003) observed in a 62 year old patient. There is a close relationship between the two organisms: *L. innocua* and *L. monocytogenes* (Hui *et al.*, 1994; Johnson *et al.*, 2004) and *L. innocua* has been identified as sharing some characteristics with *L. monocytogenes* (Liu, 2006). These include being catalase positive and motile. Moreover, it is distinguishable from *L. monocytogenes* by the difference in arylamidase activity in API Identification system (Johnson *et al.*, 2004) and the absence of some important virulence factors found in *L. monocytogenes* (Liu, 2006). However, Volokhov *et al.* (2007) and Johnson *et al.* (2004) identified in an atypical *L. innocua*, a gene cluster analogue to the pathogenicity island which is also present in pathogenic *L. monocytogenes.* Because of shared this similarities *L. innocua* has been used in many studies as a model for *L. monocytogenes* (Kamat & Nair, 1996; Doyle, 1999; Dykes *et al*., 2003; Olasupo *et al.*, 2004; Friedly *et al*., 2008; Sommers *et al*., 2008). However, compared to other *Listeria* species and *L. innocua*, *L. monocytogenes* has been implicated in many cases of food-borne infection in the past decades (CFSAN, 2001). Koo *et al.* (2011) developed a detection method that is capable of isolating between pathogenic *L. monocytogenes* and nonpathogenic *Listeria* species (Dworkin *et al.*, 2006) to aid in specific detection of this pathogen. However, Farber *et al*. (1991) discovered nonmotility and incapability to penetrate intestinal cells in nonpathogenic

L. monocytogenes strains. Chen *et al*. (2011) has recently sequenced a complete genome of nonpathogenic *L. monocytogenes.*

1.3.2 LISTERIA MONOCYTOGENES

L. monocytogenes is a Gram positive cocco-bacillus measuring 0.4-0.6 µm x 0.5-2.0 µm, grows well on a variety of laboratory media with colonies 0.2-0.8 mm in diameter. The organism is weakly β-haemolytic on blood agar (Dimaio, 2000; Kells & Gilmour, 2004; Liu, 2006), catalase-positive, feebly motile at 37°C and very motile at 25°C (Ryser & Marth, 1991; Mims *et al*., 2004). Cells are arranged in single units or in short chains appearing in palisades and may sometimes be coccoid, or they may also occur as elongated rods or long filaments appearing as bacilli (Robinson *et al*., 2000). Selective enrichment procedures followed by plating on selective agar media are required for *L. monocytogenes* isolation because, although widely distributed in the environment, it may exist in low numbers in food or other samples along with high numbers of competing microorganisms (Ryser & Marth, 1991; Bauwens *et al*., 2003; Kells & Gilmour, 2004). *L. monocytogenes* is a known pathogenic psychrotroph, implicated in many cases of food-borne infections and also the cause of listeriosis (Hui *et al.,* 1994; FDA /CFSAN, 1996; Post, 1996; Rodriguez-Lazaro *et al.,* 2004; Schlech *et al.*, 2005; CDC, 2009; Jemmi & Stephan, 2006; Chau *et al*., 2010). *Listeria* outbreaks were often associated with consumption of raw milk and unfermented dairy products, but in 1983 an outbreak of listeriosis due to the consumption of pasteurised milk was reported in Massachusetts (Ryser & Marth, 1991, Robinson *et al.,* 2000; Ryser & Marth 2007). Soft cheese made from raw milk was implicated in two outbreaks that were observed in France in 1995 and in 1997, and one outbreak in Sweden in 2001 (Lunden *et al.,* 2004). Several other outbreaks due to pasteurised milk and dairy

product consumption have been reported on different occasions (McLauchlin, 1990; Elcuaz *et al.,* 1996; Robin *et al*., 2006; Ryser & Marth, 2007; CDC, 2009).

1.3.3 PATHOGENESIS AND CLINICAL SYMPTOMS

Ingestion has been proposed as the major mode of transmission of *Listeria*, but other routes have been mentioned (Greenwood *et al*., 1997). Most healthy persons who are infected often asymptomatic or suffer only mild symptoms that may resolve quickly. The incubation period was previously thought to vary from a few days to a few weeks, but it is now known that in some cases it may be as short as one day after the consumption of heavily contaminated food (Eley, 1992). The infective dose of *L. monocytogenes* is debatable as it is believed to vary with the strain of the organism and susceptibility of the human host (Hamrick *et al*., 2003; Johnson *et al*., 2004). According to Robinson *et al.* (2000), ingestion of less than 1 000 cells is sufficient to cause disease. However, while it is assumed by other authors that from cases contracted through consumption of raw or contaminated pasteurised milk, less than 1 000 organisms/ml may cause disease only in immunocompromised persons (Todar, 2003; McLauchlin *et al*., 2004). In cheese that contained 10^3 - 10^4 organisms/g of food, no human illness was reported after consumption (Jay, 1992). This may be due to the low pH in cheese which inhibits growth of *L. monocytogenes*, and reduce the organism count to become a significant factor in minimising infection (Hui *et al.,* 1994).

Once *Listeria* is ingested it secretes an invasin which enables it to penetrate the epithelial lining of the host cells. Normally the immune system eliminates the infection before it spreads. However, *L. monocytogenes* may invade the gastrointestinal epithelium and eventually end up in the host's phagocytes where it has the ability to survive (Vardar-Unlu

et al., 1998). If the immune system is compromised, systemic disease may develop (Todar, 2003). Once the bacterium enters the host's monocytes, macrophages, or polymorphonuclear leukocytes, it may cause septicaemia. It also produces a haemolysin, *Listeriolysin O* (*LLO*) as one of the virulence factors. *LLO* is a 58 kD (kilo Dalton) protein encoded by the *hlyA* gene and is only found in virulent strains (Greenwood *et al*., 1997; Todar, 2003; Liu, 2006; Robin *et al*., 2006). Gekara *et al.* (2010) observed a block in T cell proliferation by the *LLO* virulence factor present in pathogenic strains. Other toxins produced include a cytolysin and enzymes which protect the bacteria from harmful effects of reactive oxygen radicals (Greenwood *et al*., 1997). Its intracellular presence in phagocytic cells may also permit access to the brain and transplacental migration to the foetus in pregnant women (Dimaio, 2000). The manifestations of invasive listeriosis include septicaemia, meningitis, and encephalitis. Non-invasive forms with gastrointestinal symptoms such as nausea, vomiting, and diarrhoea may proceed to more serious forms of listeriosis or these may be the only symptoms expressed (Dimaio, 2000; Todar, 2003; Lunden *et al*., 2004). Common symptoms of *Listeria* infection include back pain, muscle pain, fever, sore throat and headaches (Greenwood *et al*., 1997; Bell & Kyriakides, 1998; Dimaio, 2000; Lunden *et al.,* 2004; Liu, 2006). Alonzo *et al.* (2011) recently described some strains of *L. monocytogenes* which have acquired an enhanced ability to target and invade the heart tissue thereby causing cardiac infections.

1.3.4 GROUPS AT RISK OF INFECTION

The presence of pathogenic microorganisms in milk emerged as a major public health concern especially among those most susceptible to listeriosis. Milk containing *L. monocytogenes* can be especially dangerous to people with weakened immune systems (Dimaio, 2000; Liu, 2006). A weakened immune system can be caused by

amongst others AIDS, diabetes, kidney disease, organ transplant patients, cancer treatments and glucocorticosteroid medications (Bell & Kyriakides, 1998; FSIS/USDA, 2001; Rodriguez-Lazaro *et al.*, 2004; Goulet *et al*., 2006). With an increasing immunocompromised population due to HIV/AIDS, the risk multiplies. Healthy immunocompetent individuals are not often at risk for developing a serious listeriosis infection (Dimaio, 2000; Smith *et al.,* 2003; Liu, 2006). However, other studies have demonstrated that immunocompetent individuals who in particular consume large amounts of raw milk and dairy products were at an increased risk of developing listeriosis especially when the foodstuff was heavily contaminated with the organisms (Ryser & Marth, 1991).

Additional underlying factors that have been reported in association with listeriosis include sarcoidosis, otitis, asthma, ulcerative colitis and aplastic anaemia (Eley, 1992; Bell & Kyriakides, 1998). Age has also been shown to be a predisposing factor in listeriosis. Approximately 11% case-fatality rate has been documented in persons age 40 or younger, while a 63% case-fatality rate has been recorded for persons over the age of 60 (Eley, 1992). Age related reasons for increased incidence of listeriosis may include a decline of the immune system as a function of age, increased prevalence of immunosuppressive disorders, and increased dependence on medications that suppress the immune system (Eley, 1992). Although the conditions above may predispose patients to acquiring listeriosis, it should also be noted that persons showing no apparent immuno-compromising conditions have also been shown to acquire listeriosis. It has been noted that patients who have used cimetidine, antacids or laxatives or any other medications that decrease or neutralise gastric secretion are more likely to develop infection upon

consumption of contaminated food, indicating that the gastric acid has a protective effect against infection (Eley, 1992; Dimaio, 2000).

Another group at risk are pregnant women. According to the United States Centre for Disease control, pregnant women are 20 times more likely than other healthy adults to get listeriosis. (CDC,2005). Pregnant women who consume products contaminated with *Listeria*, are more susceptible to acquire the disease as hormonal changes during pregnancy have an effect on the mother's immune system that leads to an increased susceptibility to listeriosis as well as other diseases (Hui *et al*., 1994). During pregnancy, selective factors of cell-mediated immunity (CMI) become suppressed to prevent rejection of the foetus by the mother. However, suppression of these selective factors may result in decreased maternal resistance to *L. monocytogenes* infections and thereby increase the maternal or foetal risk to listeriosis (Hui *et al*., 1994; Smith *et al.*, 2003). Another contributing factor is that plasma levels of hydrocortisone increase to levels three to seven times during pregnancy. Cortisones are known to suppress both lymphokine activation and phagocytic activity of macrophages (Eley, 1992). In pregnancy, listeriosis occurs most often during the third trimester of pregnancy, probably due to a further decrease in immunity as compared to the early trimesters. Outcomes may be an asymptomatic maternal infection and a resulting infected infant, a severely ill mother who enters premature labour and delivers a stillborn or a severely ill infant, or death of the mother and an unaffected infant. In most cases of listeriosis, the mother is usually mildly affected presenting with flu-like symptoms, but the neonates are often severely affected. Transplacental infection in the early stages of neonatal infection results in a syndrome known as granulomatosis infantisepticum, which is a necrotic disease of the internal organs (Eley, 1992). Spontaneous abortion of the foetus is common or it may result in

stillbirth of the foetus (Eley, 1992; Dimaio, 2000; Stepanovic *et al*., 2007). Low levels of immunoglobulin-M (IgM) and decreased activity of the classical complement pathway during the neonatal period also occur and demonstrates the importance of opsonisation in the immune response to *Listeria* (Eley, 1992; Hui *et al*., 1994).

1.3.5 *LISTERIA* INFECTION IN ANIMALS

Listeriosis is also common in animals, with cattle, sheep and goats most frequently afflicted by this disease. The most common syndrome of listeriosis in animals is encephalitis, leading to observations of nervous system involvement in cattle and sheep (Bauwens *et al*., 2003; Wojciech *et al.,* 2004; Steele, 2008). Afflicted animals have been observed as disorientated, causing them to circle endlessly in one direction. For this reason listeriosis in animals is often referred to as “circling disease”. Infected animals displaying symptoms of listeric infection may excrete *L. monocytogenes* in their milk, blood and faeces, and high excretion rates of *L. monocytogenes* in milk and stools from asymptomatic cows and goats have been reported (Eley, 1992; Dimaio, 2000; Bauwens *et al.,* 2003; Ryser & Marth, 2007). Listeric infections can give rise to mastitis and *L. monocytogenes* has been isolated from milk and udders of cows with mastitis (Ryser & Marth, 1991). Bundrant *et al.* (2011) reported an outbreak in America of listeriosis in dairy cattle caused by *L. monocytogenes* serotype 4b strain.

1.3.6 EPIDEMIOLOGY

Because of the abundance of *L. monocytogenes* in nature humans are frequently exposed to this organism. It is also estimated that approximately 5% of healthy humans harbour *L. monocytogenes* in their gastrointestinal tract (Eley, 1992). Animal and human carriers have also been described (IFT, 2004) rendering either humans, an infected animal or the

farm environment as sources of contamination (Van Kessel *et al*., 2004). While asymptomatic carriage of the organism in the gut as normal flora is common, the pharynx, vagina and cervix are other sites for potential carriage (Dimaio, 2000; Stepanovic *et al*., 2007). *L. monocytogenes* is frequently shed in stools of healthy humans who show no signs of illness (Schonberg & Gerigk, 1991). The organism has been recovered from approximately 5-15% of normal adult stool samples and from 25% of household contacts of clinically ill patients (Greenwood *et al*., 1997; Mims *et al.,* 2004; Ryser & Marth, 2007). *L. monocytogenes* may also be disseminated throughout a food production plant by workers. Kerr *et al*. (1993) conducted a survey of 99 food production workers and found that 12% carried *Listeria* of which 7% was *L. monocytogenes*. However, among the 75 clerical workers used as a control, none of them harboured *Listeria* on their hands. In a study done by Weber *et al.* (1993) *L. monocytogenes* was isolated from 33% of faecal samples from the farm cattle. Prior to the 1980s, listeriosis was mainly implicated in veterinary cases, where it was associated with abortions and encephalitis in sheep and cattle and in only a few cases humans were involved (Hui *et al.*, 1994; International Commision on Microbiological Specifications for Foods, 1996; Dimaio, 2000). Listeriosis in humans is sporadic but may also appear in epidemic forms throughout the world (Dimaio, 2000; Stepanovic *et al*., 2007). The first case of listeriosis in humans was confirmed in 1929 by Nyfeldt. *Listeria* was later confirmed as a cause of meningitis and perinatal infections in 1933 and 1934 in the United States of America. However, human listeriosis remained a rare disease compared to other reportable diseases as only 36 cases of human listeriosis were recorded since it was recognised until 1945 (Ryser & Marth, 1991).

Outbreaks of listeriosis are usually due to a common source of contaminated food which served as a vehicle. In earlier outbreaks raw milk was suspected of being the mode of

transmission but without scientific proof (Lunden *et al*., 2004). Increased awareness of listeriosis, as a result of many such major outbreaks led to improved methods of detecting this organism. This resulted in a dramatic increase in the number of reported cases since 1985 and the incidence was said to have increased 10-fold in the United States of America. The same trend was observed in data collected in Europe where the incidence rates increased dramatically from 1949 to 1987. Additional reports of *L. monocytogenes* in several outbreaks have also been reported from other countries, such as Egypt, Iran, Turkey, Morocco and South Africa (Ryser & Marth, 2007). The CDC (2009) reported an outbreak of human disease caused by *L. monocytogenes* in the United States in which pasteurised milk was implicated. Food recalls due to listeria contaminated foodstuffs are also on the rise (News Desk, 2012). Epidemics of *Listeria* usually occur in the community (Elcuaz *et al.,* 1996) but hospital associated outbreaks have also been reported in organ transplants and neonatal units (Fredericksen & Samuelsson*,* 1992; Fernandez-Sabe *et al*., 2009). Clinical listeriosis is catergorised into two groups: infection in males and non-pregnant females, and infection in the pregnancy and their neonates. Greenwood *et al.* (1997) reported that pregnancy and neonatal disease account for 30-45% of cases of listeriosis. In a study done in Britain, 34% of listeriosis cases were observed during pregnancy (McLauchlin, 1990). The Food-borne Diseases Active Surveillance Network (FoodNet) conducted a study and observed the highest reported incidence of listeriosis in children yonger than four years (CDC, 2010). In non-pregnant adults, the incidence of infection increased with age. Refer to Table 1.2 for a summary of some outbreaks of listeriosis contamination.

Table 1.2 Outbreaks of listeriosis

YEAR	COUNTRY	FOOD SOURCE	REFFERENCE
1949 - 1957	Germany	Raw milk	Seeliger, 1961
1979	Boston	Lettuce	Schlech *et al*., 1983
1979	Canada	Cabbage	Schlech *et al*., 1983
1981	Canada	Coleslaw	Schlech *et al*., 1983
1983	Boston, Massachusetts	Whole and 2% Pasteurised milk	Fleming *et al*., 1985
1985	Los Angeles	Mexican style cheese	Linnan *et al*., 1988
1983 -1987	Switzerland	Soft cheese	Bula *et al*., 1995
1997	Illinois	Milk	Dalton *et al*., 1997
2000	Northern Italy	Corn	Aureli *et al*., 2000
2001	Sweden	Soft cheese	Carrique-Mas *et al*., 2003
2001	North Carolina	Mexican style cheese	CDC (2001)
2009	United States	Pasteurised fluid milk	CDC (2009)
2009	Denmark	Beef	Smith *et al*., 2011
2010	Norway	Camembert cheese	Johnsen *et al*., 2010

Allerberger & Wagner (2010) reported that a higher rate of listeriosis is seen in patients older than 65 years.Mook *et al.* (2011) reported an increase of listeriosis cases in England and Wales for patients 60 years of age and older. However, other countries have

observed declines in listeriosis cases. For example, in France a study showed a decline in listeriosis cases from 51% to 24% over a period of ten years in all ages (Goulet *et al*., 2006).

1.3.7 PREVALENCE OF *LISTERIA* IN FOOD

Various foods of vegetable and animal origin have been implicated as sources of infection as many cases of listeriosis have been directly related to a food source contaminated with *L. monocytogenes* (Pearson & Marth, 1990; Eley, 1992; FSIS/USAD, 2001; Shetty *et al*., 2009). Raw milk was believed to be responsible for another human listeric infection that was seen in West Germany between 1949 and 1957 (Seeliger, 1961; Ryser & Marth, 1991; Lunden *et al*., 2004; Ryser & Marth, 2007). However, the first scientifically proven food related listeriosis was reported in 1981, where the source was coleslaw (Lunden *et al*., 2004; IFT, 2004). *L. monocytogenes* has been associated with foods such as cheese, raw vegetables, salads, raw and cooked poultry, raw meats of all types, seafood, eggs and raw and smoked fish (Hayes *et al*., 1986; Jersek *et al*., 1999; Inoue *et a*l., 2000; Wojciech *et al*., 2004; Gouws & Liedemann, 2005; Sofos, 2005; Kirkan *et al*., 2006; Laciar *et al*., 2006; Millet *et al*., 2006; Thevenot *et al*., 2006; De Santis *et al*., 2007; Aarnisalo *et al.,* 2008; Garrido *et al*., 2009; Garrido *et al*., 2010; Gebretsadik *et al*., 2011; Jakobsen *et al*., 2011; Wang *et al*., 2012). Refer to Table 1.3 for food products that have been associated with *Listeria*.

Table 1.3 Food associated with *Listeria* contamination

FOOD	ORGANISM	REFERENCE
Meat salads, French salad, beef steak, chicken, cheese	*L. monocytogenes*	Jersek *et al*., 1999
Salted herring herring salads	*L. monocytogenes* and *L. innocua*	Dąbrowski *et al.,* 2000
Mince meat, salmon, raw seafood	*L. monocytogenes*	Inoue *et al*., 2000
Steamed chicken, Turkey, minced meat	*L. monocytogenes*	Wojciech *et al*., 2004
Mince, patty, trout, fish, salad, cheese salad	*L. monocytogenes*	Gouws & Liedemann, 2005
Frankfurters	*Listeria*	Sofos, 2005
Helix pomatia	*L. monocytogenes*	Kirkan *et al*., 2006
Cabbage, lettuce, squid	*L. monocytogenes*	Laciar *et al*., 2006
Mussel, hake, mackerel	*L. innocua*	Laciar *et al*., 2006
Raw milk, cheese	*L. monocytogenes*	Millet *et al*., 2006
Raw pork meat	*L. monocytogenes*	Thevenot *et al*., 2006
Sheep milk cheese	*L. monocytogenes*	De Santis *et al*., 2007
Raw milk, cheese, ice cream, yoghurt, butter	*L. monocytogenes* *L. innocua* and *L. seeligeri.*	Rahimi *et al*., 2010

Raw beef, egg, raw milk cheese	*L. monocytogenes* *L. innocua, L. murrayi,* *L. welshimeri , L. grayi* and *L. seeligeri*	Gebretsadik *et al*., 2011
Bovine cheese	*L. monocytogenes*	Jakobsen *et al*., 2011
Cold-smoked salmon	*L. monocytogenes*	Chitlapilly *et al.,* 2011
Turkey meat	*L. monocytogenes*	Erol & Ayaz, 2011
Milk	*L. innocua*	Cagri-Mehmetoglu *et al*., 2011

There are several reports where *Listeria* contaminated raw milk, pasteurised milk and other products made from raw milk have also been incriminated in outbreaks (Hayes *et al.,* 1986; Vardar-Unlu, 1998; Lunden *et al*., 2004; Millet *et al*., 2006; CDC, 2009). Following an outbreak of listeriosis *L. monocytogenes* was again recovered from 12% of milk samples and 14% of milk equipment in a study on raw milk (Hayes *et al.,* 1986). A high prevalence was also seen in meat. In a study done in Gauteng, South Africa, 66% of chicken carcasses were found to be contaminated by three organisms of which *L. monocytogenes* was found in 19% of the carcasses (Van Nierop *et al*., 2005).

1.3.8 SEROTYPING AND NUCLEIC ACID TECHNIQUES

The genus *Listeria* has for many years been known to consists of only one species, *L monocytogenes*. Later *L. grayi, L. murray, L. ivanovii, L. welshimeri, L. seeligeri* and *L. innocua* were identified (Ryser & Marth, 1991*).* Two recently reported species are *L. rocourtiae* (Leclercq *et al*., 2010) and *L. marthii* (Graves *et al.,* 2010*).* Serotyping was the

first method used to differentiate between the strains. Strains are serotyped according to their variation in the somatic (O) and flagellar (H) antigens due to virulence variation between the serotypes (Jersek *et al*., 1999; Liu, 2006). Five DNA related groups were devised from *L. monocytogenes* strains. Group 1 includes strains belonging to pathogenic serovars 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, and 7. Group 2 is assigned to all haemolytic strains belonging to serovar 5. Group 3 contains non-haemolytic and non-pathogenic strains of serovars 4ab, 6a, and 6b. Group 4 contains serovars 6a and 6b. Group 5 haemolytic and non-pathogenic strains serovars 4c, 4d, 6b and undesignated serovars. Groups are also divided into Lineages or Divisions I, II and III based on genetic content and evolution. Lineages are again divided into subgroups based on the similarities of highly conserved genes and specific markers (Borucki & Call, 2003; Doumith *et al*., 2004; Ryser & Marth, 2007; Lopez *et al*., 2008).

Approximately 16 serotypes of *L. monocytogenes* have already been identified. The serotypes 4b, 1/2a, 1/2b and 1/2c are the most common clinical isolates and are responsible for 90 - 98% of human infections (Eley, 1992; Jersek *et al*., 1996; Wojciech *et al*., 2004; Laciar *et al*., 2006). Furthermore, while *L. monocytogenes* 4b serotype is isolated mostly during epidemic outbreaks, serotype 1/2a and 1/2b are associated with sporadic outbreaks (Borucki & Call, 2003; Liu, 2006; Yi Chen & Knabel, 2007; Pichler *et al*., 2009). Between 1984 and 1985, 650 raw milk samples were analysed and 12.6% contained *Listeria* species with *L. monocytogenes* found in 4.2% of samples (Ryser & Marth, 2007). Of the *L. monocytogenes* isolated from milk, 16 were serotype 1 and 10 were serotype 4. Although serological confirmation is not routinely done for regulatory identification of *L. monocytogenes*, it is useful for determining the prevalence of specific serotypes in epidemiological studies and for environmental recontamination tracking.

Genetic methods of detection are increasingly being employed in diagnostic and research fields. Although they may be considered expensive in terms of cost and skilled personnel, it is at the discretion of each institution to weigh cost against quality. For effective prevention and control of disease it is essential to use rapid, specific and sensitive tests for diagnosis of food-borne pathogens to shorten the time of analysis (Suwansonthichai & Rengpipat, 2003; Sukhadeo & Trinad, 2008; Ross *et al*., 2009). PCR is well established as a research method for the detection of *Listeria* in food and has been successfully used in numerous studies (Jacquet, 1992; Ramos, 1998; Holko *et al*., 2002; Rodriguez-Lazaro *et al*., 2004; Van Kessel *et al*., 2004; Gouws & Liedemann, 2005; Rudi *et al.*, 2005; Kirkan *et al*., 2006; De Santis *et al*., 2007; Yi Chen & Knabel, 2007; Aurora *et al*., 2009; Rossmanith & Wagner, 2010; Traunsek *et al*., 2011). PCR has also been used to identify *Listeria* species for diagnostic purposes (Siggens, 1995; Greenwood *et al*., 1997; Bubert *et al.,* 1999; Todar, 2003; Rodriguez-Lazaro *et al*., 2004; Liu, 2006; Robin *et al*., 2006).

PCR serotyping has been a preferred method and has been successfully used to identify the four major serotypes 1/2a, 1/2b, 1/2c and 4b (Ericsson, 1995; Borucki & Call, 2003; Doumith *et al*., 2004; De Santis *et al*., 2007; Yi Chen & Knabel, 2007; O'Connor *et al*., 2010). Since molecular methods have been developed to reduce the analysis time and to increase specificity, recent developments in DNA analysis have allowed for even better differentiation of strains with a higher discrimination power (Jersek *et al*., 1999; Kaclikova *et al*., 2001). Such methods include DNA fingerprinting, which has been used to characterise and separate bacterial strains that possess the same bacteriophage type or serotype. This can be done by comparing the banding patterns of DNA fragments in an agarose gel. Development of highly discriminatory typing systems has provided a method

of distinguishing among related species and of detecting variation among strains within species (Eley, 1992).

Repetitive element based subtyping has been used to differentiate between genetically related strains (Judd *et al*., 1993; Jadhav *et al.*, 2012). It has also been successfully used to subtype and differentiate between *L. monocytogenes* serotypes. Repetitive element based subtyping is a PCR typing method that incorporates the use of primers based on short extragenic repetitive sequences. These sequences are typically present at many sites around the bacterial chromosome. Because the number and location of the repetitive sequences are quite variable, the number and size of the inter-repeated fragments generated can similarly vary from strain to strain (Ryser & Marth, 2007). Examples of such sequences are the ERIC sequence and the REP sequence and have been used successfully in different studies (Judd *et al*., 1993; Jersek *et al*., 1996; Jersek *et al.*, 1999; Ventura *et al*., 2003; Wojciech *et al*., 2004; Laciar *et al*., 2006). The differences in band sizes are used to characterise isolates and differentiate between isolates.

Unlike serotyping, which has a limited value as an epidemiological tool, ERIC-PCR has been successfully used to generate DNA fingerprints that allow for better discrimination between bacterial strains (Jersek *et al.*, 1999). Fingerprinting has been used successfully in outbreaks to distinguish between involved isolates (Pichler *et al*., 2009). Employment of such molecular typing methods is beneficial as they offer the advantage of a higher discrimination power and can allow the isolate to be characterised to species and strain level. As a result of better laboratory detection techniques, new food-borne pathogens continue to be identified.

1.4 CONCLUSION

Control of *L. monocytogenes* is particularly difficult in terms of chill-storage (Eley, 1992) since it can survive at low temperatures. This was also observed by Glass & Doyle (1989) where food stored at 4.4°C showed survival and multiplication of *L. monocytogenes*. Conditions of prolonged cold storage are advantageous to proliferation of *L. monocytogenes*. Consequently it should be suspected that all raw milk may be contaminated with food-borne bacteria or specifically with *Listeria*, and adequate measures to ensure proper pasteurisation and also to prevent post-pasteurisation contamination of processed dairy products must be adhered to by dairy manufacturers to ensure a safe product (Vardar-Unlu *et al*., 1998; Lunden *et al.,* 2004). Since refrigeration of raw milk creates selective conditions for the growth of psychrotrophic bacteria (Beales, 2003), psychrotrophs such as *Listeria* in dairy products is an issue that requires serious attention There exist great differences in pathogenic potential among strains of *L. monocytogenes* and useful information can be obtained from typing (Rocourt *et al.*, 2000). This will help to identify and differentiate the organisms (Lukinmaa *et al.*, 2003) in order for measures to be taken during an epidemic to contain the infection and prevent further spread once the source has been identified. The incidence of food poisoning has to be documented and reported if improved public health is a concern for the health authorities. Factors such as the frequency of food poisoning in relation to causative food product and location of the causative food product may prove to be beneficial for control and prevention of outbreaks and epidemiological surveys.

1.5 PROBLEM STATEMENT

The biological nature and nutritional quality of foods support the growth of microorganisms. These microorganisms are often a result of contamination especially by the spoilage bacteria, which may result in food-borne illness (Arakawa *et al*., 2008). When evaluating the quality of refrigerated milk and dairy products, the concern is almost exclusively with psychrotrophic and spoilage bacteria and control of these organisms during processing as well as in the finished product is cumbersome as refrigeration does not restrict or eliminate the growth of psychrotrophs (Becker *et al.*, 2000; Rowe, 2003; Gandhi & Chikindas, 2007).

Food safety failures usually receive much public attention that leads to demands for increased product testing (Hobbs & Roberts, 1993). However, in some developing countries, such as Lesotho, not much attention is given to food safety failure due to the lack of product testing and inspection. In addition to this, industries often cannot afford to implement quality assurance processes. In Maseru (Lesotho), there is only one large scale dairy producer and several informal producers sell raw bovine milk to the dairy. However, some producers sell raw milk directly to the community. Unlike this large scale dairy producer where the manufacturer utilises control measures to ensure food safety, the situation is different with the majority of informal milk producers who supply raw milk which has not been tested for pathogens. Milk producers who neglect milk quality standard procedures consequently pose a threat to the general health of the consumers.

HACCP system is currently employed in Lesotho from FAO/WHO and food safety guidelines have to be implemented with regard to food inspection and monitoring. Commercial dairy products produced from raw milk are subjected to performance

guidelines which involve prevention and control of contaminants that will compromise the quality. Any producer should, therefore, strive to work with sanitised equipment and in a sanitised environment to avoid contamination (Rowe, 2003). At the same time, large scale producers as well as informal producers are faced with the challenge to prevent recontamination of the food (Schonberg & Gerigk, 1991). Recontamination is often the result of contamination from food contact surfaces, or within equipment (Bower *et al.*, 1996; Simoes *et al.,* 2010). There is no known documented or published information regarding the incidence of *L. monocytogenes* and the quality of milk produced by the informal farmers in the Maseru area as well as prevalence of foodborne illnesses as a result of inadequate services that facilitate food safety and disease surveillance.

1.6 AIMS AND OBJECTIVES

The aim of this study was to investigate whether milk produced and consumed in the Maseru area meets the specified dairy standard with regard to the presence of *L. monocytogenes* and total bacterial counts.

The objectives of the project were to:

1. Determine the bacterial quality of milk in the study area (Maseru).
2. Determine the prevalence of *Listeria monocytogenes* in milk.
3. Determine the molecular characterisation of *Listeria monocytogenes.*
4. Determine the prevalence and perceptions on milk and/or milk products consumption by the community.

CHAPTER II

METHODOLOGY

2.1 SAMPLE COLLECTION

Two hundred samples were collected for this study. Hundred and sixty unpasteurised milk samples from local farmers were collected at the dairy production company reception area of the dairy plant in Maseru, Lesotho. This was the milk brought in by local producers from Maseru area. Approximately 15 samples were collected per week and analysed over a period of three months. Samples were collected in sterile 50 ml bottles and directly aliquoted into sterile labelled screw cap test tubes. Samples, at room temperature, were immediately transported to the dairy plant laboratory for microbial analysis. The time from collection to analysis in the laboratory was approximately one hour (h) or less. Microbial analysis was performed at the dairy plant. Permission was granted by the dairy management to carry out analysis on unpasteurised milk samples at the laboratory of the dairy production company in Maseru, Lesotho.

Fourty fresh pasteurised milk samples were bought from different local shops in Maseru at intervals of 10 samples per week. All the samples were within their stipulated expiry period. Samples were transported on ice to the laboratory and analysis was performed on the day of purchase. The time from collection to analysis in laboratory was approximately three hours. Microbial analysis on the pasteurised milk samples and confirmation of presumptive *Listeria* isolates were carried out in the research laboratory at the Central University of Technology, Free State, South Africa.

2.2 GEOGRAPHIC STUDY AREA

The Maseru constituencies from which milk samples were collected at the dairy were Maseru, Stadium Area, Mabote, Motimposo, Tšosane, Lithabaneng, Lithoteng, Abia and Qoaling (Figure 2.1).

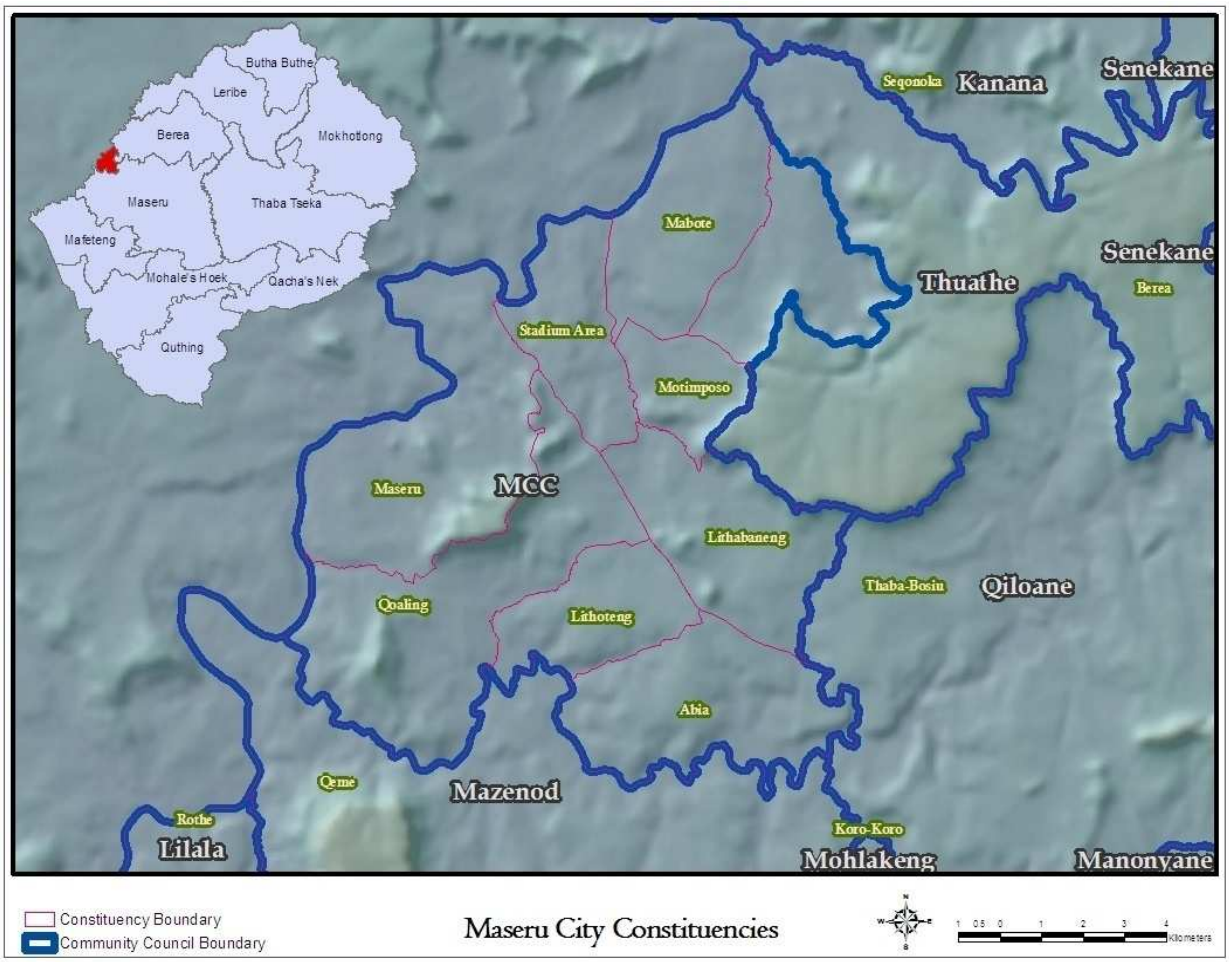

Figure 2.1: Maseru city constituencies under study

2.3 ISOLATION AND IDENTIFICATION

All procedures were followed according to the operating standards as stipulated by regulations relating to milk and dairy products in South Africa incorporated in Act 54 of

1972, the Foodstuffs, Cosmetics and Disinfectants Act, in R. 1555 of 21 November 1997 (South Africa, 1997).

2.3.1 TOTAL AEROBIC PLATE COUNT

Petrifilm™ Total aerobic plate count (Merck, SA) was used to determine the total number of aerobic bacteria present in a sample. Milk samples (raw and pasteurised) were diluted in Ringers solution (Oxoid, England) to 10-fold serial dilutions of 10^{-3} and 10^{-4} dilutions for each sample to be analysed, using a sterile pipette, one millilitre (ml) was dispensed onto the centre of petrifilm for each dilution, and the inoculum was evenly distributed and spread over the entire petrifilm plate area using the provided spreader. The plates were left undisturbed for one minute to allow for gel formation and then incubated aerobically at ±37°C for 24h after which colonies were counted from each film. Tests were performed in duplicate and the average of the two readings multiplied by the dilution factor was taken as the total count.

2.3.2 TOTAL COLIFORM COUNT AND TOTAL *E. COLI* COUNT

The Chromocult agar (Merck, SA) was used to determine the total coliform and *E. coli* counts in raw and pasteurised milk. The chromogenic substrates in the agar allow for the simultaneous detection of total coliforms and *E. coli*. The substrate X-glucuronide is used for the identification of ß-D-glucuronidase activity, which is characteristic for *E. coli. E. coli* cleaves both Salmon-GAL and X-glucuronide, and positive colonies take on a dark blue to violet colour. In order to confirm *E. coli* colonies, KOVACS' indole reagent was used. Suspected *E. coli* colonies were cultured in peptone water and then incubated aerobically for 24 h at 37°C. Few drops of KOVACS' indole reagent were added to the cultures. A positive indole formation confirmed the presence of *E. coli*. The characteristic enzyme

produced by coliforms bacteria, ß-D-galactosidase, cleaves the Salmon-GAL substrate and causes the salmon to red colour of the coliform colonies. The medium was inoculated by spreading 1 ml of the sample material (from serial dilutions) on the surface of the plates and then incubated aerobically for 24 h at 35-37°C. Total coliform count was recorded as the sum of red colonies (coliforms) and dark-blue colonies (*E. coli*). Each sample was done in duplicate and the average of the two readings multiplied by the dilution factor was taken as the total count.

2.3.3 PHOSPHATASE TEST

The Aschaffenburg and Mullen phosphatase test was used to determine the pasteurisation status of the milk. Disodium p-nitrophenyl phosphate is used to detect colur change in the presense of alkaline phosphatase. The method was applied as stipulated in Regulation 1555 of 1997 (South Africa, 1997). The buffer was prepared from 0.3% (w/v) anhydrous sodium carbonate (Merck, SA) and 0.15% (w/v) sodium bicarbonate (Merck, SA) dissolved in 100 ml distilled water. To 100 ml of the buffer, 150 mg of disodium p-nitrophenyl phosphate (Merck, SA) was added and dissolved. The solution was freshly prepared every day before use.

Samples were incubated for 30 min in a waterbath set at 25°C. In a sterile tube, 5 ml of the phosphate buffer just the buffer or the solution with the disodium p-nitrophenyl phosphate and 1 ml of warmed pasteurised milk sample were added using sterilised pipettes and mixed by shaking. The tubes were incubated in a water bath for 2 h at 37°C and colour changes observed after every 10 min. Bacterial phosphatase is used as a marker of pasteurisation in the dairy industry. Pasteurisation denatures alkaline phosphatase in milk. In the event where milk is unpasteurised it turns to a bright yellow

colour within the first 5 min, while pasteurised milk will not show any colour change. Pasteurised milk which turned bright yellow after 10-20 min was recorded as having 'bacterial phosphatase' (due to high bacterial counts). Samples that remained white or turned pale yellow after 30 min were recorded as pasteurised. Raw milk was used as a positive control sample and boiled milk was used as a negative control sample (was heated for 3 minutes at 100°C).

2.4 ISOLATION OF *LISTERIA*

Isolation of *Listeria* was carried out using *Listeria* enrichment broth (LEB, Merck, SA), Fraser broth (Merck, SA) and *Listeria* selective agar (Merck, SA) in accordance with the International Organisation for Standardisation (ISO) 11290-1 (1996) and standard 143:1990 of the Food and Drug Administration/International Dairy Federation) for milk and milk products for the detection of *L. monocytogenes*. Biochemical tests and analytical profile index (API) *Listeria* (bioMérieux, SA) were used for confirmation.

2.4.1 TWO-STAGE ENRICHMENT METHOD

Selective enrichment for *Listeria* was attained by using the two-step enrichment method which promotes the growth of *Listeria* and inhibits accompanying bacteria as described by Post (1996). One ml of the milk samples was inoculated into 10 ml *Listeria* enrichment broth (Merck, SA) and incubated aerobically for 24 h at 30°C. This was followed by the second enrichment step where 0.1 ml from the first cultures was inoculated into 10 ml of Fraser *Listeria* selective enrichment broth (Merck, SA) and incubated aerobically at 35°C for 24 h. When blackening of the broth was observed**,** Oxford *Listeria* selective agar (Merck, SA) was subcultured with 50 µl of the culture broth and incubated aerobically at 35°C for up to 24-48 h. *L. monocytogenes* hydrolyses esculin to esculetin and forms a

black complex with iron (III) ions, and produces brown-green coloured colonies with a black halo. All presumptive colonies were selected for further testing.

2.4.2 IDENTIFICATION OF *LISTERIA*

Sub culturing was done on *Listeria* selective agar to produce single colonies of pure cultures. Culture purity was confirmed by morphological examination of isolated colonies (observation of brown-green coloured colonies with a black halo). Gram stain and biochemical tests such as catalase, oxidase and motility tests were performed on all presumptive *Listeria* species prior to API confirmation to eliminate any false positive *Listeria*-like organisms. All isolates which were Gram positive cocco-bacilli or bacilli, catalase positive, oxidase negative and motile at 25°C were selected for further biochemical analysis using the API specific for *Listeria*. Sub-cultures were prepared on blood agar for confirmation and differentiation with API *Listeria* (bioMérieux, SA) according to the manufacturer's instructions. Refer to Figure 2.2 for the order followed in *Listeria* isolation.

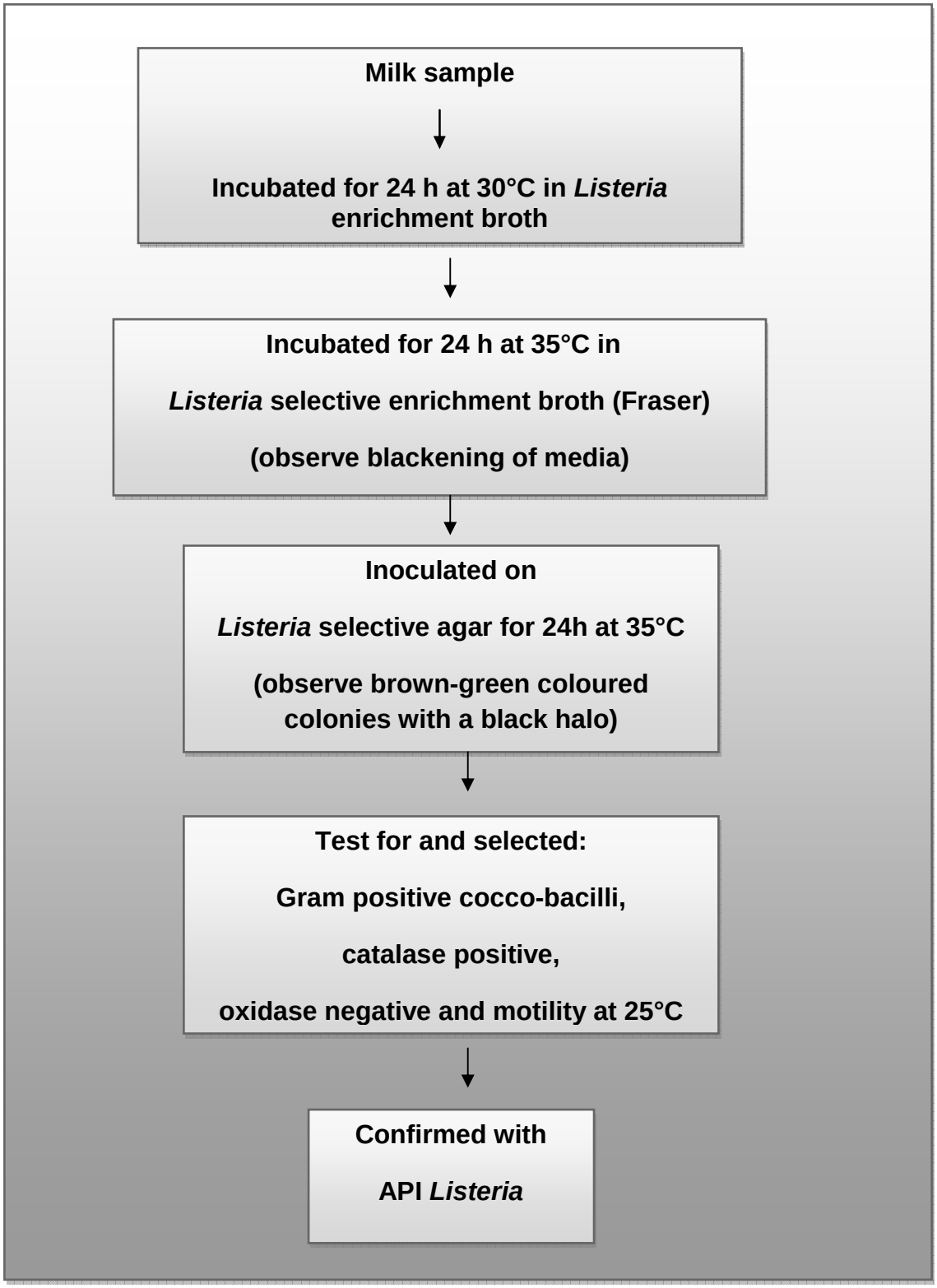

Figure 2. 2 Flow diagram for the isolation of *Listeria* species.

2.5 MOLECULAR ANALYSIS

2.5.1 DNA EXTRACTION AND QUANTIFICATION

The strains confirmed by API as *Listeria* were stored at -70°C in Trypticase soy broth (Merck, SA) with 15% sterile glycerol. Bacterial growth was reactivated by subculturing onto 5% sheep blood agar plates and aerobically incubated overnight at 37°C. Growth on blood agar was re-suspended in 500 µl of 1x Tris-EDTA buffer, vortexed and boiled for 10 min in a waterbath to lyse the cells and release the cell contents. The samples were centrifuged (10 000-12 000 rotation per minute for 10 minutes) and the supernatant, which contained the DNA material were transfered into new micro centrifuge tubes. Extracted DNA samples were stored at -20° until further analysis (Borucki & Call, 2003). The sediment which contained the cell debris was discarded.

Five microliters (µl) of DNA and 1 ml of sterile distilled water were vortexed in an Eppendorf tube, transferred to a cuvette and dsDNA concentration was determined with a Helios Epsilon spectrometer (USA) set to 260 nm. The initial concentration and the optical density (OD) of each sample were recorded. Based on the respective OD readings, samples were further diluted (using the formula $C_1V_1 = C_2V_2$) with sterile distilled water to achieve the approximate optical density of 0.005 which corresponds to final concentration of 50 ng/µl for each DNA sample to be used in PCR. DNA concentration (ng/µl) = OD x DILUTION FACTOR x 50 (International Genetically Engineered Machine, 2008)

2.5.2 PRIMER DESIGN FOR *LISTERIA MONOCYTOGENES*

The primers were designed from the *LLO* gene, the complete coding sequence (cds) from the GenBank accession number EU073158 of *L. monocytogenes* was downloaded from

the National Centre for Biotechnology Information website (NCBI, 2008). To verify primer specificity the BLAST program from the same website was used and the ClustalX program was used for multiple alignments of sequences. Primers were designed to amplify a 654 bp length part of the gene. The forward primer was taken as the 18 base forward sequence (CCTAAGACGCCAATCGAA) from base 243 and the reverse primer was taken as the inverted complement of the 18 base sequence (AAGCGCTTGCAACTGCTC) from base 879. The melting temperature (T_m) was determined using a program from Promega website (Tm calculation for oligonucleotides, 2006). Primers for *L. innocua* were used as was proposed by Bubert *et al.* (1999).

2.5.3 PCR METHODOLOGY FOR *LISTERIA MONOCYTOGENES*

For the identification of *LLO* gene (encoded by *hlyA*), specific primers forward primer for *L. monocytogenes* (LM1F) and reverse primer for *L. monocytogenes* (LM1R) were used for identification of a 702-bp fragment (Siggens, 1995). The second primer set, LM2F and LM2R designed in this study, was also used to amplify a 654-bp fragment of the gene. Refer to Tables 2.1 and 2.2 for the reaction conditions and mixture.

Table 2.1 Reaction conditions for *Listeriolysin O* gene amplification

Name of oligonucleotide	**Sequence (5'-3')**		**Source**
LM1F	TGCAGTGACAAATGTGCC		Siggens, 1995
LM1R	CCGTATGCCACACTTGAG		
LM2F	CCTAAGACGCCAATCGAA		Designed for this study
LM2R	AAGCGCTTGCAACTGCTC		
Step	**Temp.**	**Time**	**Cycles**
Initial Activation	95°C	5 min	1
Denaturising	95°C	1 min	30
Annealing	54°C	1 min	
Elongation	72°C	2 min	
Final Extension	72°C	5 min	1

Table 2.2: Reaction mixture for *LLO* gene amplification

Reagent	**Concentration (final)**	**Volume per tube (µl) for a total of 25 µl tube**
PCR buffer (Promega,USA)	1x	3
$MgCl_2$ (Promega, USA)	2.5 mM	3
dNTP (dATP, dCTP, dTTP, dGTP) (Promega,USA)	200 µM (each)	0.06
Primer (IDT,USA)	25 pmols (each)	0.25
Taq polymerase (Promega,USA)	0.25U	0.05
DNA	62.5ng	1.25

2.5.4 PCR METHODOLOGY FOR *LISTERIA INNOCUA*

Amplification was done with primers Ino 2 (5'ACTAGCACTCCAGTTGTT 3') and Lis1B (5'TTATACGCGACCGAAGCC3") which identify all serotypes of *L. innocua* for the 870-bp DNA fragment of the *iap* gene (Bubert *et al.*, 1999). Refer to Tables 2.3 and 2.4 for the reaction conditions and mixture.

Table 2.3: Reaction conditions for *L. innocua iap* gene amplification

Name of oligonucleotide	**Sequence (5'-3')**		**Source**
Ino 2	ACTAGCACTCCAGTTGTT		Bubert *et al.*, 1999
Lis1B	TTATACGCGACCGAAGCC		
Step	**Temp.**	**Time**	**Cycles**
Initial Activation	95°C	5 min	1
Denaturising	95°C	15 seconds(s)	30
Annealing	58°C	30 s	
Elongation	72°C	50 s	
Final Extension	72°C	8 min	1

Table 2.4: Reaction mixture for *L. innocua iap* gene amplification

Reagent	**Concentration (final)**	**Volume per tube (µl) for a total of 25 µl tube**
PCR buffer	1x	3
$MgCl_2$	1.5 mM	3
dNTP (dATP, dCTP, dTTP, dGTP)	200 µM (each)	0.09
Primer	100 ng of each	0.18
Taq polymerase	1.5 U	0.3
DNA	50 ng	1

2.5.5 SEROTYPING

A multiplex PCR was done with D1, D2, FlaA and GLT primers to determine four different serovars of *L. monocytogenes* which are identified as fragments lengths of 214bp, 140bp, 538bp and 483bp respectively (Borucki & Call, 2003). Refer to Tables 2.5 and 2.6 for the reaction conditions and mixture.

Table 2.5: Reaction conditions for *L. monocytogenes* serotyping

Name of oligonucleotide	**Sequence (5'- 3')**		**Amplicon size (bp)**
D 1F	GGATATTTTATCTACTTTGTCA		
D 1R	TTGCTCCAAAGCAGGGCAT		214
D 2F	GCGGAGAAAGCTATCGCA		
D 2R	TTGTTCAAACATAGGGCTA		140
FlaA 1F	TTACTAGATCAAACTGCTCC		
FlaA 1R	AAGAAAAGCCCCTCGTCC		538
GLT 1F	AAAGTGAGTTCTTACGAGTTT		
GLT 1R	AATTAGGAAATCGACCTTCT		483
Step	**Temp.**	**Time**	**Cycles**
Initial activation	95°C	3 min	1
Denaturising	95°C	30 s	30
Annealing	50°C	30 s	
Elongation	72°C	60 s	
Final extension	72°C	10 min	1

Table 2.6: Reaction mixture for *L. monocytogenes* serotyping

Reagent	Concentration (final)	Volume per tube (µl) for a total of 25 µl tube
PCR buffer	1x	6
$MgCl_2$	2.5 mM	3
dNTP (dATP, dCTP, dTTP,dGTP)	0.2 µM (each)	0.06
Primer	50 ρmols (each)	0.5
Taq polymerase	1.0 U	0.2
DNA	50 ng	1

2.5.6 GENOTYPING

ERIC 1 and ERIC 2 primers were used to type *L. monocytogenes* strains using the ERIC-PCR method (Laciar *et al.*, 2006). Refer to Tables 2.7 and 2.8 for the reaction conditions and mixture.

Table 2.7: Reaction conditions for *L. monocytogenes* genotyping

Name of oligonucleotide	Sequence (5'- 3')		
ERIC 1	ATGTAAGCTCCTGGGGATTCAC		
ERIC 2	AAGTAAGTGACTGGGGTGAGCG		
Step	**Temp.**	**Time**	**Cycles**
Initial activation	95°C	5 min	1
Denaturising	90°C	30 s	30
Annealing	50°C	30 s	
Elongation	52°C	1 min.	
Final extension	72°C	8 min	1

Table 2.8: Reaction mixture for *L. monocytogenes* genotyping

Reagent	Concentration (final)	Volume per tube (µl) for a total of 50 µl tube
PCR buffer	1x	3
$MgCl_2$	1.5 mM	3
dNTP (dATP, dCTP, dTTP,dGTP)	0.2 mM (each)	0.06
Primer	1 mM (each)	0.3
Taq polymerase	1.0 U	0.2
DNA	100 ng	1

2.5.7 GEL ELECTROPHORESIS

All amplification products were resolved in a 1.5% low melting (LM) Sieve agarose gel (Sigma) in 1x TAE buffer (40mM Tris-acetic acid and 1mM EDTA, pH 8.3). An aliquot of 18 µl of the PCR product was loaded with 4 µl 6x loading dye (15% ficoll, 0.03%, bromophenol blue, 0.03 % xylene cyanol FF, 0.4% orange G, 10mM Tris) (Promega, USA). An aliquot of 6 µl of the 100bp DNA molecular weight marker (Promega, USA) mixed with 12 µl 1x TAE buffer and 4 µl 6x loading dye was used to confirm the size of the products. The gels were run at 80 Volts for 1 hour 40 min. The gels were stained with 0.5 mg/ml ethidium bromide solution (Jersek *et al*., 1999; Gouws & Liedemann, 2005; Laciar *et al*., 2006; De Santis *et al*., 2007; Yi Chen & Knabel, 2007). The PCR products were then visualised under UV light and photographed.

2.6 QUESTIONNAIRE

2.6.1 DEVELOPMENT OF QUESTIONNAIRE AND DESIGN

As part of the larger study, a questionnaire was used to investigate the incidence of milk and/or milk products consumption by the households and to investigate the demographic and socio-economic characteristics of the consumer. It was also important to determine whether the consumer perceive these products to be the cause of any illness. The preparatory stage involved the selection of the sample design and design of the questionnaire. The sample survey method was determined as the appropriate technique for data collection in this study. Sampling procedure was a stratified two-stage cluster sample.

There are 192 308 people living in Maseru City Council (MCC) (Lesotho Statistical Yearbook, 2010) and 44, 300 households (Bureau of Statistics, 2006). According to this information the average household size for Maseru City is 4.3. MCC consists of seven constituencies which are further divided into enumeration areas which are residential areas (Bureau of Statistics, 2006). The enumeration areas can be further stratified into low, middle and high-income areas based on the level of development in each area.

The cluster sample comprised of developed planned areas, semi-unplanned areas, and unplanned area households. Households were from the selected residential areas (villages) within the constituencies of the MCC area. A sample was drawn from each residential area by sampling with probability proportional to size with the number of households as a measure of size. Taking into consideration a fact of density per area, in constituencies with households numbering less than 10 000, 5 villages were selected, while in constituencies with more than

10 000 households 10 villages were selected. A sample size of 5% of households per selected villages was taken.

2.6.2 SAMPLE SIZE AND RESIDENTIAL AREAS

Forty-five (45) residential areas were identified within Maseru. A representative sample of three hundred (300) households were selected as samples within these areas and according to different income levels, high income households (48) were considered as those from well-planned areas, while middle-income households (113) were considered as those from semi-planned areas; and low-income households (139) were considered as those from low-cost, unplanned areas according to the MCC area plans. Refer to Table 2.9 for villages and households sampled.

Table 2.9: Number of villas and households sampled per Constituency

CONSTITUENCY	Selected number of villages	Number of households sampled	Low income households	Mid income households	High income households
MOTIMPOSO	5	30	18	12	0
STADIUM AREA	5	30	0	18	12
MASERU CENTRAL	10	68	38	0	30
QOALING	10	70	49	21	0
LITHOTENG	5	35	21	14	0
LITHABANENG	5	37	7	30	0
ABIA	5	30	6	18	6
TOTAL	**45**	**300**	**139**	**113**	**48**

Quick household listing at site was used to identify households to be interviewed i.e. if the number of households to be intervied was 20 from a total of 100 households, (100/20 = 5), every fifth household will be selected counting from the first household on the list in that area. In cases where there was no response from households, a replacement was made with the next neighbouring household.

2.6.3 QUESTIONNAIRE DESIGN

A questionnaire was designed with the help of a statistician to capture both demographic and consumption details of all the respondents.It was an interviewer administered questionnaire. Each respondent was personally interviewed and their informed consent was requested after a short introduction to explain the purpose of the questionnaire. The interviewees were assured of confidentiality.

The information requested from the participants was to obtain:

- Information about village, age, gender, marital status, and educational background and occupation.
- Milk and/or milk products consumed (fresh (pasteurised) milk, raw milk, commercial sour milk, yoghurt or cheese)
- Symptoms experienced after consumption (nausea, vomiting, diarrhoea, abdominal pain, headache, fever)
- Incubation period (time before symptoms are experienced)
- Causative food product (participants perception)
- Action taken (i.e. medical attention)

Questionnaires were completed over a period of one month. The results were analysed and tabulated. Refer to Figure 2.3 for the sample of the questionnaire.

CHAPTER III

MICROBIAL CONTENT IN THE MILK

3.1 INTRODUCTION

The majority of bacteria in milk are non-pathogenic. However, these organisms are of particular concern to the dairy industry because they affect product quality. Producers cannot sell milk of unacceptable standards to the public and legislation prohibits them from doing this. Monitoring the microbial quality of milk is compulsory and can serve to help a dairy producer identify inefficiencies in the production of milk and to improve quality. Levels of microorganisms and pathogens have to comply with legislation and regulations (Millet *et al.*, 2006), and the food industry must implement a variety of effective control measures to limit potential hazards. These include testing the milk for the presence of indicator organisms.

Faecal coliforms are often used as an indicator of faecal contamination and the potential risk of zoonotic pathogens (Yánez *et al*., 2006). However, coliforms such as non-pathogenic *E. coli* are present in the digestive tract of animals and the presence of these faecal coliforms is assumed to be indicative of the presence of other pathogenic organisms. Faecal coliforms can contaminate raw milk through faecal contamination as a result of poor milking practices, by udder infection or the external milking environment (Ryser & Marth, 1991). The total bacterial count is therefore used in the dairy industry as an indication of milk quality.

3.2 BACTERIAL COUNTS

The total aerobic plate count, total coliform count and *E. coli* count for 160 raw milk samples and 40 pasteurised samples were determined. The phosphatase test was also carried out to determine the pasteurisation state of the 40 pasteurised milk samples.

3.2.1 RAW MILK TOTAL AEROBIC PLATE, TOTAL COLIFORM AND *E. COLI* COUNTS

The total aerobic counts of 73 (45.6%) of the raw milk samples ranged from 10^4 – $2x10^5$ cfu/ml, which is within the acceptable range. However 87 (54.4%) of the samples had counts greater than 200 000 cfu/ml (Figure 3.1a). Refer to Chapter 1 (Table 1.1) for standard bacterial count values for milk. Coliform counts were performed in both the pasteurised and raw milk samples. Out of all the raw milk samples 71 (44.4%) had coliforms counts below 20 cfu/ml while 89 (55.6%) had coliform counts higher than 20 cfu/ml. From the raw milk samples tested in this study 125 (78.1%) complied with the standard count for total *E. coli* while 35 samples (21.9%) had counts greater than 10 cfu/ml (Figure 3.1b). Figures 3.2 a & b reflect the percentage values for aerobic, coliform and *E.coli* counts respectively.

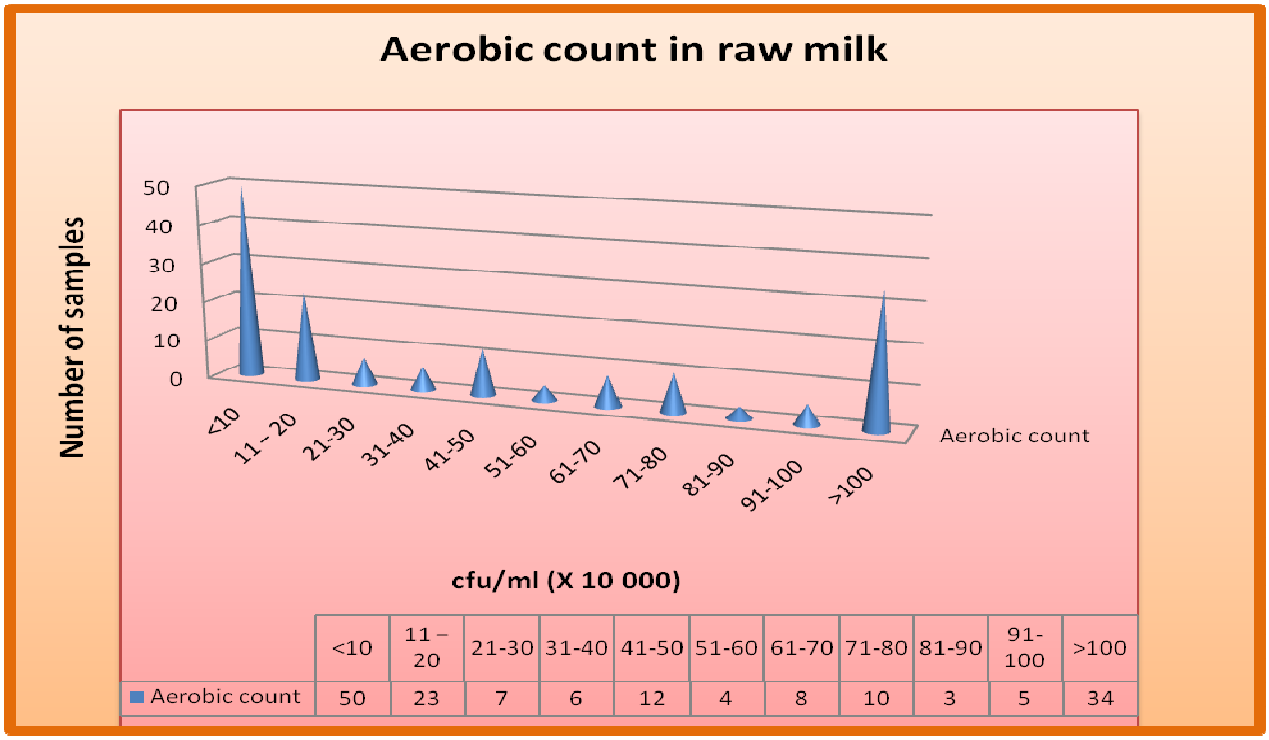

Figure 3.1(a): Total aerobic count in raw milk

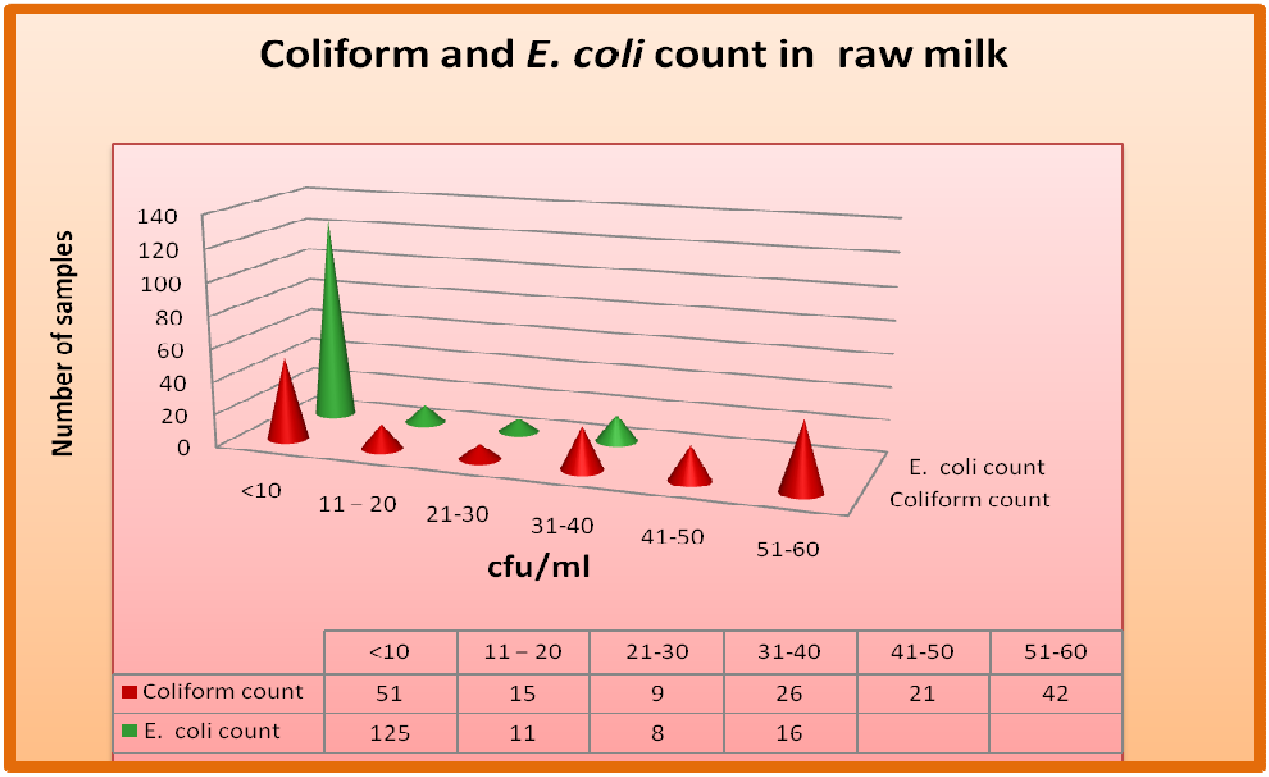

Figure 3.1(b): Total coliform and *E.coli* in raw milk

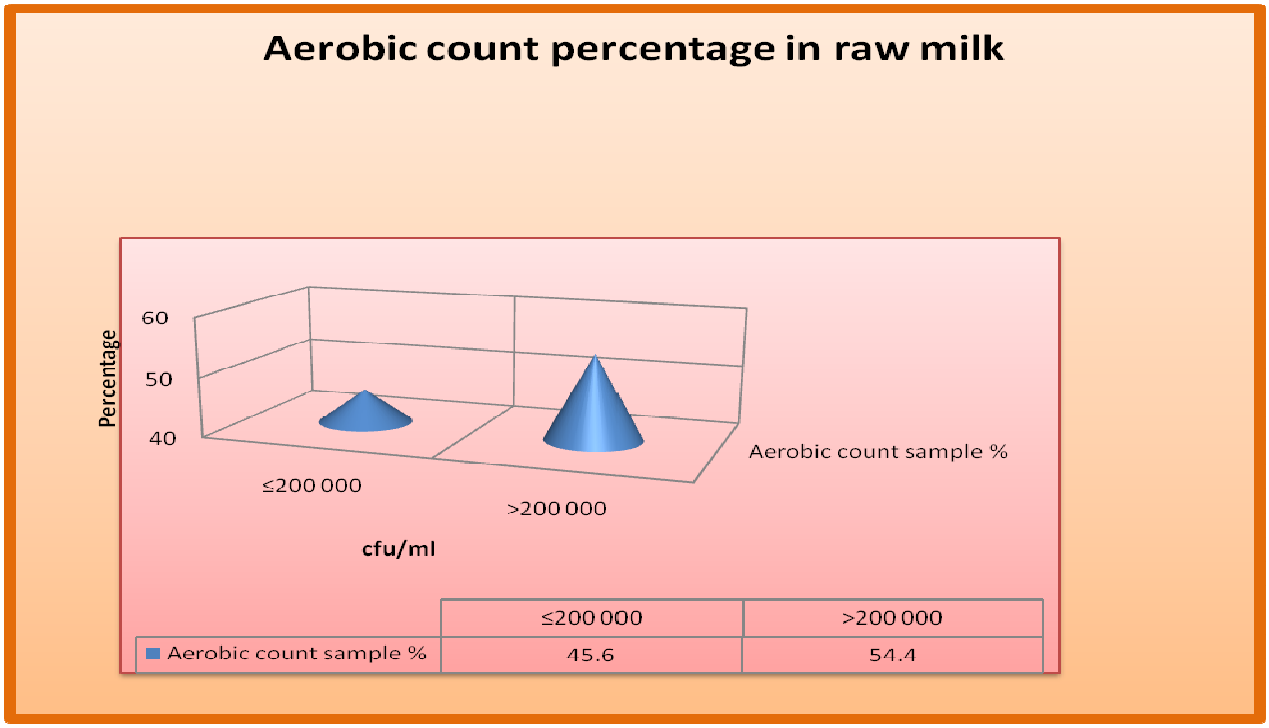

Figure 3.2(a): Total aerobic percentages in raw milk

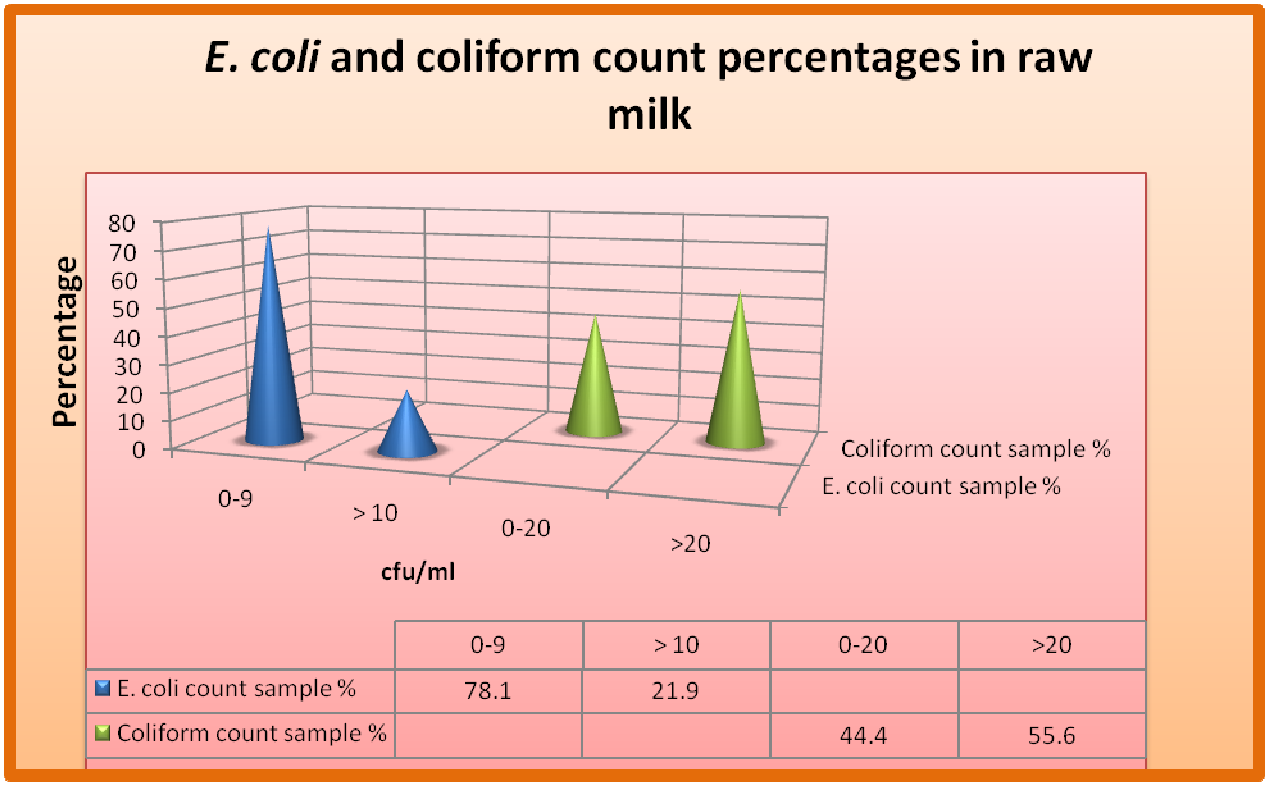

Figure 3.2(b): Total coliform and *E.coli* percentages in raw milk

3.2.2 PASTEURISED MILK TOTAL AEROBIC PLATE, TOTAL COLIFORM AND TOTAL *E. COLI* COUNTS

With regard to the pasteurised milk samples, only 13/40 (32.5%) of these samples were within the acceptable range (50 000 cfu/ml) of the national standard while 27/40 (67.5%) were above the acceptable range (Figure 3.3a). This shows that most of the pasteurised milk samples exceeded the national standard for total aerobic plate counts in pasteurised milk sold directly to the public. In pasteurised milk 33 out of 40 samples had total coliform counts of ≤ 20 cfu/ml while 7 out of 40 had higher counts. *E. coli* was absent in 1 ml for tested factory pasteurised milk samples (Figure 3.3b). Only 17.5% of the pasteurised samples showed non-conformance (Figure 3.4b) compared to 55.6% from the raw milk samples. Bacterial phosphatase was detected in 7/40 of the pasteurised samples.

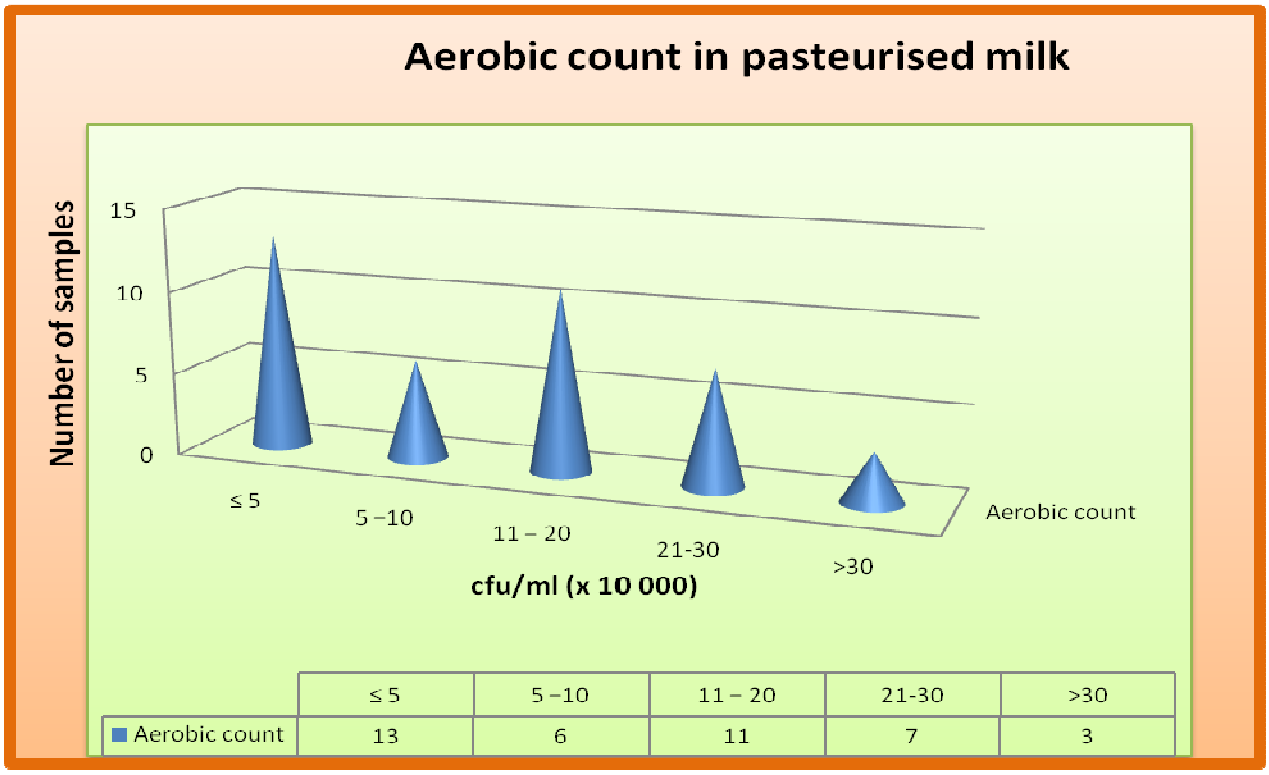

Figure 3.3(a): Total aerobic plate count in pasteurised milk

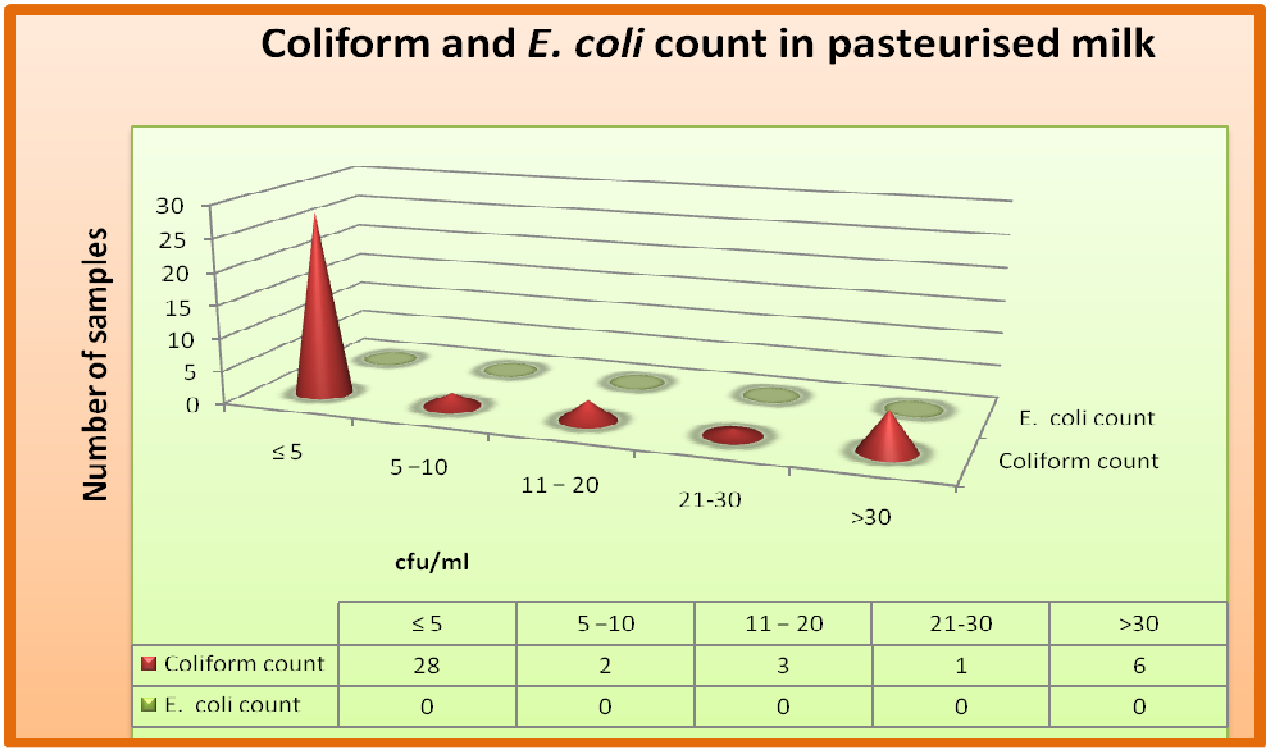

Figure 3.3(b): Total coliform and *E.coli* count in pasteurised milk

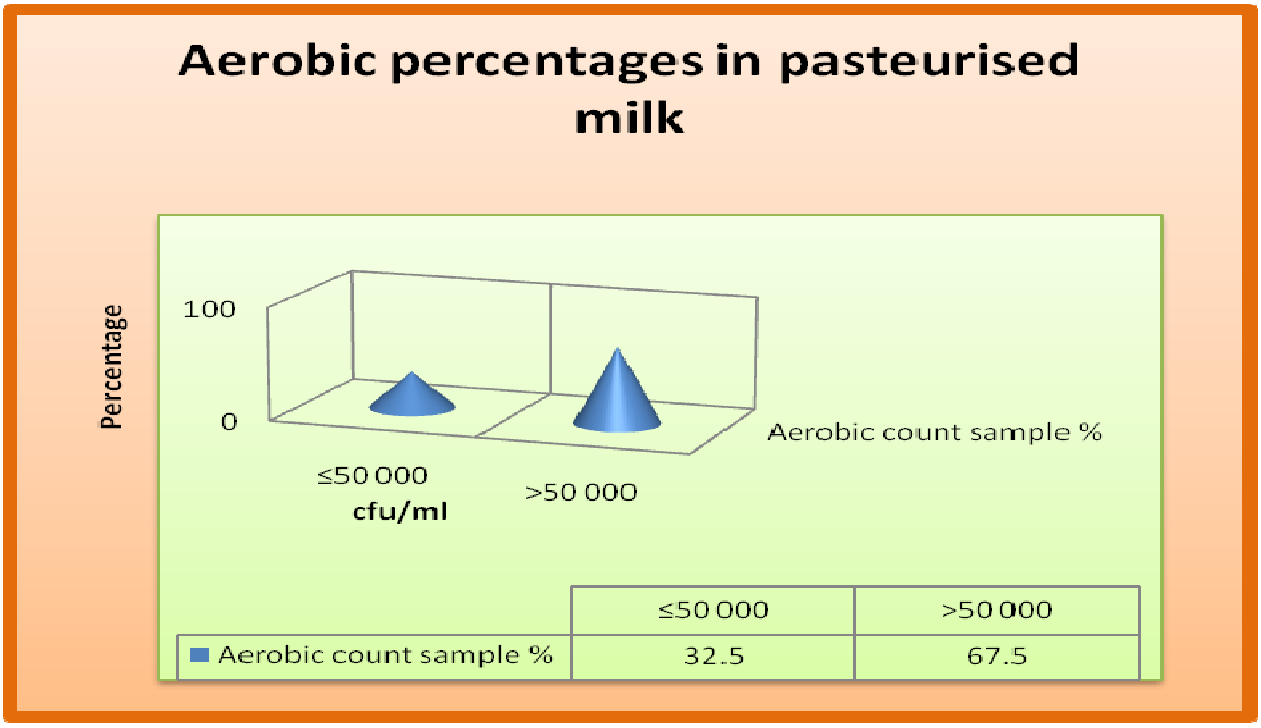

Figure 3.4(a): Total aerobic count percentages in pasteurised milk

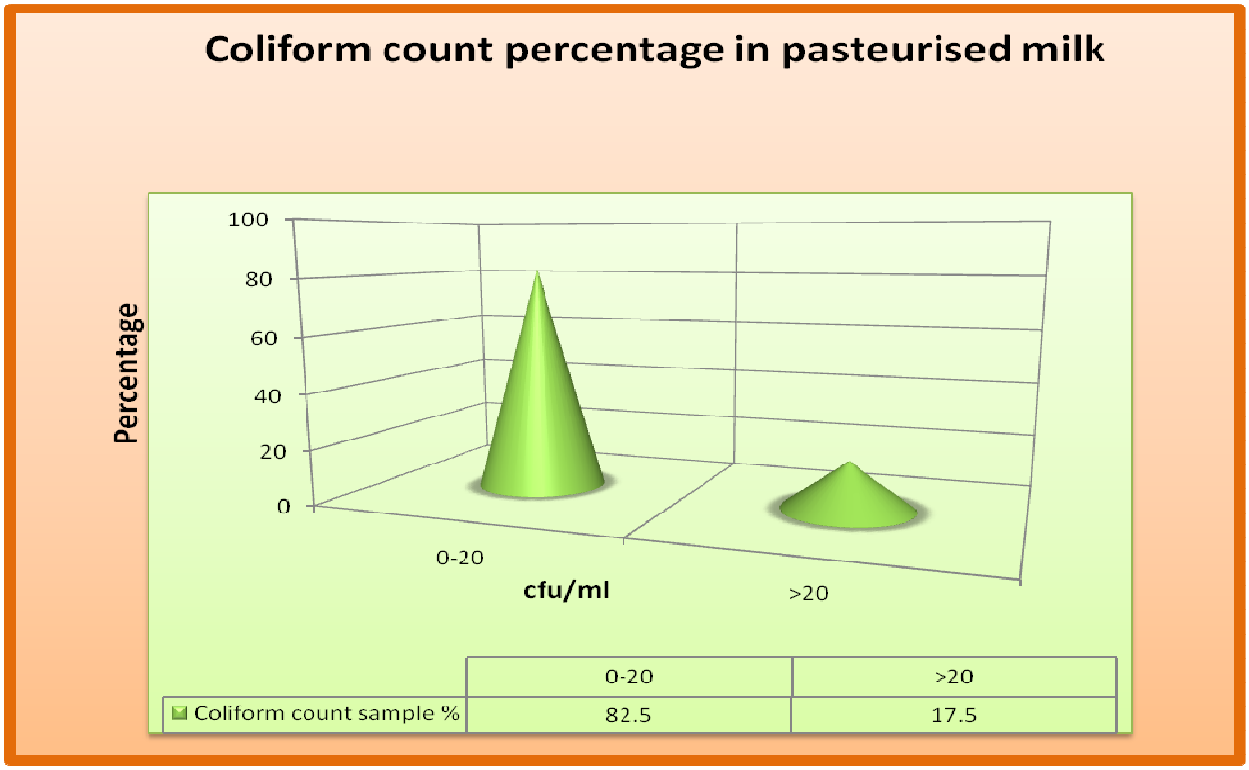

Figure 3.4(b): Total coliform count percentage in pasteurised milk

3.3 PREVALENCE OF *LISTERIA*

3.3.1 BIOCHEMICAL TESTS

Biochemical test results and API were used to identify and confirm *Listeria* isolated from raw milk. Twenty nine of the 200 samples were presumptive for *Listeria.* However, only 9 (4.5%) were selected for API based on their biochemical reactions. The API confirmed six isolates to be *Listeria* species. Of the six (3.75%) *Listeria* species, five were identified as *L. monocytogenes* and one as *L. innocua*

3.4 DISCUSSION AND CONCLUSION

Dairy products may serve as vehicles of transmission for pathogenic strains. In this study the results showed that 54.4% of the raw milk samples had total aerobic bacterial counts above the stipulated range, 55.6% of the samples had high coliform counts, and, 21.9% of the samples exceeded the *E. coli* counts allowed for raw milk. Although complete elimination of coliform bacteria entering the milk is considered difficult (Bell & Kyriakides, 1998), a very low total coliform count in pasteurised milk is essential to ensure good quality milk. Of the pasteurised milk samples, 17.5% had a high coliform count (Figure 3.4b). However, the majority of the samples (82.5%) had acceptable coliform counts of ≤ 20 cfu/ml. These results are similar to the findings by Cagri-Mehmetoglu *et al.* (2011) who isolated both pathogenic *E. coli* and *L. monocytogenes* in two cheese processing environments and Van Kessel *et al.* (2004) where a low percentage of the samples tested had unacceptably high total coliform counts.

A large percentage (67.5%) of pasteurised milk samples showed unacceptably high aerobic plate counts (Figure 3.4a). This effect could be attributed to the pasteurisation process not being effective in reducing aerobic plate count of the pasteurised milk or to post processing contamination as a result of the equipment not being thoroughly clean and could have resulted in biofilm formation. All the pasteurised milk samples in this study were purchased whilst within their expiration date. However, bacterial phosphatase activity was detected in 17.5% of these pasteurised milk samples, suggesting the possibility of post pasteurisation contamination or large bacterial numbers in the sample which may result in ineffective pasteurisation. Unacceptable levels of food-borne pathogens and spoilage organisms may compromise the quality of the food product. These findings are a

reflection of the poor quality of raw milk in the study area and also indicate that some retail milk may not be suitable for human consumption.

Listeria species were detected in 6/160 (3.75%) raw milk samples, five of which were *Listeria monocytogenes* and one *Listeria innocua*. No Listeria was found in pasteurised milk samples. Previous studies have found similar rates of *L. monocytogenes* in raw milk (Ryser & Marth, 2007). In a study conducted by Waak *et al.* (2002), the prevalence of *L. monocytogenes* in raw milk was reported to be 1% while Vardar-Unlu *et al.* (1998) reported a 6% raw milk prevalence of *Listeria*.in raw milk. However, higher counts have also been reported. According to Hayes *et al.* (1986) *L. monocytogenes* was recovered from 12% of raw milk samples and Holko *et al.* (2002) found 5/40 raw milk samples to be contaminated with *L. monocytogenes.* In a study by Rahimi *et al.* (2010), the prevalence of *Listeria* in raw milk in Iran was 22.6% and 18.9% in cheese. Kells & Gilmour (2004) found 44% prevalence of *Listeria* in raw milk samples, while none was found in pasteurised milk. Compared to other studies, the prevalence of *Listeria* was low in current study. However, these pathogens represent a potential risk to consumers of raw milk and raw milk products.

There was no correlation between the high bacterial counts in some of the samples and the presence of *Listeria*. In addition to some of the *Listeria* contaminated samples revealing high counts, elevated counts were also seen in some of the non-*Listeria* contaminated samples. Having affirmed the occurrence of *L. monocytogenes* in raw milk samples, it is interesting that *L. innocua* was also identified from the analysed samples although it was not within the aims of this study. However, this organism has been found on several occasions to co-exist together with *L. monocytogenes* (Dąbrowski *et al*., 2000;

Laciar *et al*., 2006). Vardar-Unlu *et al.* (1998) reported a 4% *L. monocytogenes* and 2% *L. innocua* prevalence in raw milk in Sivas, Turkey. Dąbrowski *et al.* (2000) conducted an investigation where both *L. monocytogenes* and *L. innocua* strains were isolated from meat and seafood. The presence of these organisms in the same environmental setting is common as other researchers have found co-existence of these two organisms in their studies. Schmid *et al.* (2005) suggested that both *L*. *monocytogenes* and *L. innocua* share the same branch on the phylogenic tree of the *Listeria* genus. In a study conducted by Van Kessel *et al.* (2004) 6.5% of the milk samples in US dairies had *L. monocytogenes*. Kells & Gilmour (2004) did a survey of milk processing plants and *L*. *monocytogenes* was found in 22.2% of raw milk samples. In a study conducted by Cagri-Mehmetoglu *et al.* (2011) 50% of raw milk samples contained *L. innocua.*

The isolated *Listeria* came from the samples collected in February to April 2009. The *Listeria* contaminated samples also came from six different producers in the MCC area from the villages Lithabaneng, Leqele (Lithabaneng), Tsosane, Semphetenyane (Qoaling) and Abia, hence there is no identification of regional occurrence. The low prevalence of *Listeria* may be attributed to inhibition of growth by high levels of competing organisms present in milk. A lack of inhibition by the reagents was suspected since competitive bacteria were also cultured. Identification of suspected colonies from the media was also problematic, due to the occurrence of *Listeria*-like organisms. These organisms had the typical appearance and behaviour of *Listeria* in both the enrichment broth and selective media. As experienced by other authors, phenotypic properties by which bacteria are identified when using culture methods may not always be expressed and may be difficult to interpret (Gouws & Liedemann, 2005; Ryser & Marth, 2007). This was found by Gebretsadik *et al.* (2011) who observed cultures with similar growth and morphological

characteristics as *Listeria* but were not confirmed as *Listeria*. As bacterial adaptation to different environments causing similarities in phenotype, as well as resistance to ingredients in enrichment and selective media is often evident, conventional methods of detection cannot be exclusively relied upon, and genetic methods of detection should be included (Gouws & Liedemann, 2005). On the other hand, Besse *et al.* (2010) observed nutritional competition amongst *Listeria* species during the enrichment process. It is important to note that some samples positive for *Listeria* may go undetected due to overgrowth by natural background flora during enrichment as some strains of *Listeria* may not be able to grow competitively. Such findings highlight the importance of using molecular methods as a confirmatory technique for isolating *L. monocytogenes* as well as for identification purposes. Rossmanith & Wagner (2010) defined limitations in microbiological methods as compared to molecular techniques in pathogen detection. In a study conducted by Alessandria *et al.* (2011) more positive samples were recovered when using molecular methods compared to traditional methods. As reported by other authors, molecular methods were not only developed to reduce analysis time but also because of their high specificity in identification and characterisation among species (Bubert *et al*., 1999: Holko., 2002; De Santis *et al*., 2007).

It is important to note the risk of supplying contaminated milk to the dairy. Such milk increases the chances of post pasteurisation contamination as most of the plant equipment may easily be in contact with the milk and serve as possible sites for contamination, more especially due to formation of biofilms (Husu, 1990; Hood & Zottola, 1995). This was observed by Kells & Gilmour (2004) who isolated *Listeria* in pasteurised milk from raw milk with a 44% *Listeria* prevalence in milk processing plants. Alessandria *et al.* (2011) observed similarity of strains that were isolated from the equipment as well as

the final product when conducting molecular characterisation of *L. monocytogenes* in a dairy processing plant. Doijad *et al*. (2011) also recovered 19.52% *Listeria* isolates from equipment in three milk processing plants. The supply of poor quality raw milk to the plant will therefore impact the final product.

CHAPTER IV

MOLECULAR IDENTIFICATION

4.1 INTRODUCTION

Molecular methods of detection are increasingly being employed in diagnostic and research fields and for effective prevention and control of disease they are essential to shorten the time of conventional analysis. PCR has been used as a diagnostic method for the detection of *Listeria* in foods (Siggens, 1995; Holko *et al*., 2002; Johnson *et al*., 2004; Rodriguez-Lazaro *et al*., 2004; Van Kessel *et al*., 2004; Gouws & Liedemann, 2005; Kirkan *et al*., 2006; De Santis *et al*., 2007; Yi Chen & Knabel, 2007). For the identification of *Listeria* serotypes, PCR-serotyping has also been successfully used to identify the four major serotypes 1/2a, 1/2b, 1/2c and 4b in food samples (Borucki & Call, 2003; Doumith *et al*., 2004; Johnson *et al*., 2004; De Santis *et al*., 2007; Hamdi *et al*., 2007; Yi Chen & Knabel, 2007; Doijad *et al*., 2011). Molecular tests have also been used to characterise and detect variations among strains within species. This includes ERIC-PCR which has been successfully used to generate DNA fingerprints that allow for better discrimination between bacterial strains (Judd *et al*., 1993; Jersek *et al*., 1996; Jersek *et al*., 1999; Ventura *et al*., 2003; Wojciech *et al*., 2004; Laciar *et al*., 2006) and it has shown to be beneficial in epidemiological studies.

The six *Listeria* strains isolated from this research were confirmed by PCR by determining the presence of *LLO* gene in *L. monocytogenes* (Siggens, 1995; Greenwood *et al*., 1997; Todar, 2003; Liu, 2006; Robin *et al*., 2006) and the *iap* gene present in *L. innocua* (Bubert *et al.*, 1999; Rodriguez-Lazaro *et al*., 2004). To determine the serotype of each *L. monocytogenes* strain, PCR-serotyping was employed as a method of choice. ERIC

PCR was then used to characterise and separate bacterial strains that possessed the same serotype by determining their fingerprinting profiles.

4.2 AMPLIFICATION OF *LISTERIOLYSIN O (LLO)* GENES

Using the two primer sets previously described (Chapter 2, page 41) the isolates were analysed for the presence of *listeriolysin O* gene found in *L. monocytogenes* isolates. The reaction conditions and mixtures in Table 2.1 & 2.2 (Chapter 2 page 44) were used to produce the gels (Figure 4.1 and 4.2).

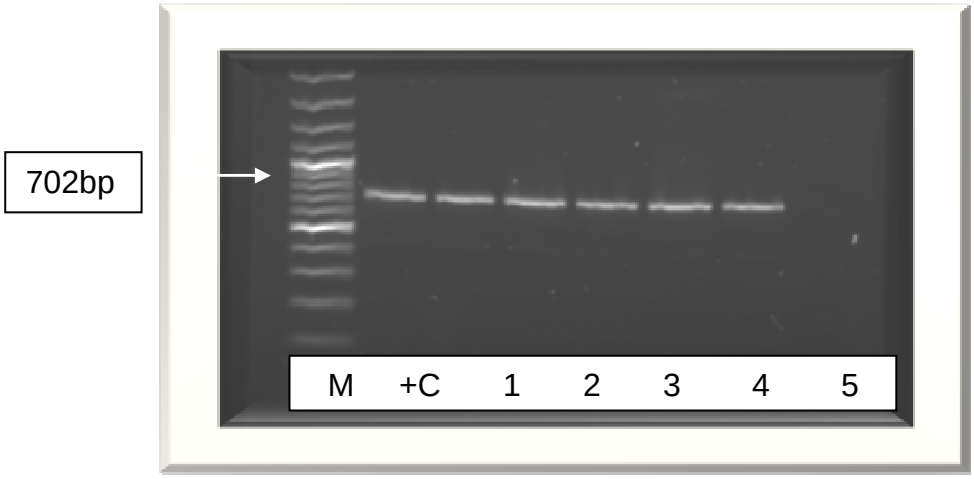

Figure 4.1: Gel-electrophoresis pattern of 702bp *LLO* gene fragment of *L*. *monocytogenes* isolates. Lane M, 100bp molecular weight marker (Madison, Promega). Lane +C, *L. monocytogenes* positive control. Lanes 1-5, amplification of isolates 27, 14, 5, 29 and 19. Lane -C represents the negative control (no DNA).

The Figures 4.1 and 4.2 show the presence of the *LLO* gene fragments in all the identified *L. monocytogenes* isolates. This confirmed the identification of the isolates. The bands on Figure 4.2 are well aligned to confirm the presence of a 645bp fragment.

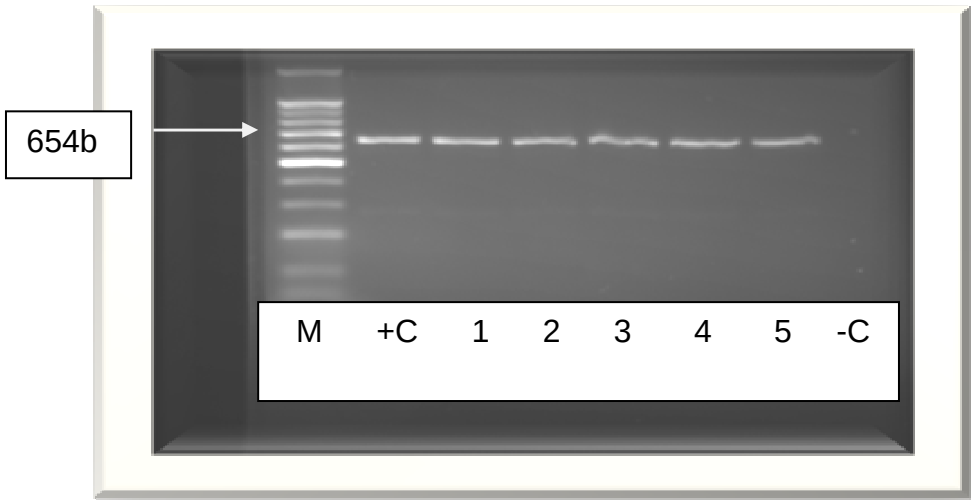

Figure 4.2: Gel-electrophoresis pattern of the 654bp *LLO* gene fragment of *L. monocytogenes* isolates. Lane M, 100bp molecular weight marker (Madison, Promega). Lane +C, *L. monocytogenes* positive control. Lanes 1-5, amplification of isolates 27, 14, 5, 29 and 19. Lane -C represents the negative control (no DNA).

Figure 4.1 also demonstrate the presence of 702bp *LLO* gene. The skewness may be due to the effect of high temperature on the gel created during the electrophoresis process.

4.3 AMPLIFICATION OF 870BP DNA FRAGMENT OF THE *IAP* GENE

Using primers described by Bubert *et al.*, (1999), the 870bp DNA fragment of the *iap* gene was amplified to confirm the organism *L. innocua*. The reaction conditions and mixtures in Table 2.3 & 2.4 (Chapter 2 page 45) were used for the reaction conditions and mixtures to produce the gel (Figure 4.3).

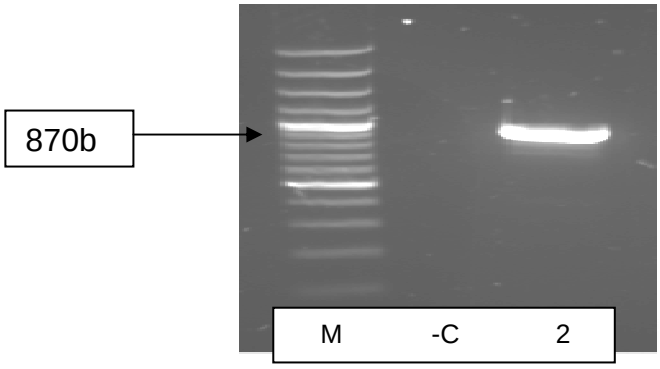

Figure 4.3: Gel-electrophoresis pattern of the 870bp DNA fragment of the *iap* gene of *L. innocua* isolate. Lane M, 100bp molecular weight marker (Madison, Promega). Lane -C, negative control (no DNA). Lane 2, amplification of isolate no.16.

4.4 SEROTYPING

A multiplex PCR assay was performed for amplification of the four serovar-specific fragments of the genus *Listeria* using the primers described by Borucki & Call (2003). The results show that strains were of the same serotype as they all had one particular fragment. The reaction conditions and mixtures in Table 2.5 & 2.6 (Chapter 2 page 46 & 47) were used for to produce the gel (Figure 4.4).

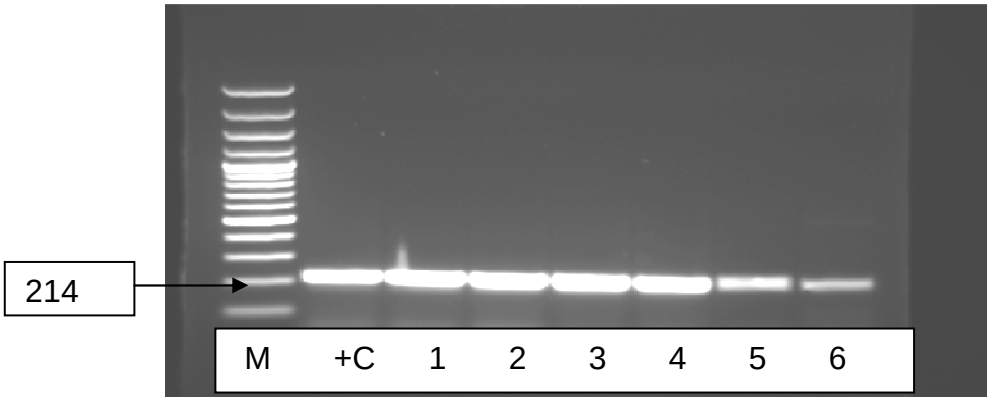

Figure 4.4: PCR amplification. Lane M, 100bp molecular weight marker (Madison, Promega). Lane +C, *L. monocytogenes* positive control organism. Lanes 1-5, amplification

of isolates 27, 14, 5, 29 and 19. Lane 6, amplification of *L. innocua* isolate16. All the strains revealed a fragment length of 214bp.

4.5 GENOTYPING

Typing of the *L. monocytogenes* strains was achieved by amplification of the enterobacterial repetitive intergenic consensus (ERIC) sequences to characterise the strains based on their banding patterns. The reaction conditions and mixtures (Table 2.7 & 2.8, chapter 2 page 47-48) were used for to produce the gel (Figure 4.5).

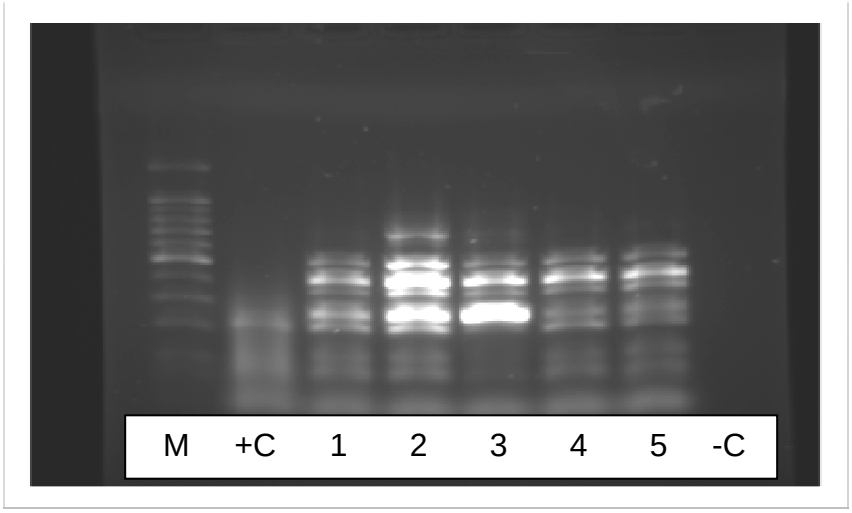

Figure 4.5 ERIC-PCR fingerprints. ERIC-PCR fingerprints of the *L. monocytogenes* strains isolated from milk. Lane M, 100bp molecular weight marker (Madison, Promega). Lane +C, *L. monocytogenes* positive control organism. Lanes 1-5, amplification of isolates 27, 14, 5, 29 and 19. Lane –C, negative control (no DNA).

4.5 DISCUSSION AND CONCLUSION

The presence of any *Listeria* species in food is regarded as significant. In this study, different primer sets were used to detect the *llo* and *iap* genes for *L. monocytogenes* and *L. innocua* respectively. The *llo* gene encodes a virulence factor of *L. monocytogenes*

Serotyping of the isolated *Listeria* strains revealed that all six isolates belonged to serotypes in Lineages I or III and can either be seroidentities 4b (or 4d, 4e), 4a/c and 1/2b (or 3b) (Ryser & Marth, 2007). The amplification of the 214bp fragment length (Figure 4.4) identifies all these serotypes but does not distinguish between them. This limitation has also been detected by other researchers (Borucki & Call, 2003; Doumith *et al*., 2004; De Santis *et al*., 2007). However, serotypes 4a, 4c and 4d are rarely isolated from food and implicated in listeriosis (Lunden *et al*., 2004; Van Kessel *et al*., 2004) while 1/2a, 1/2b and 4b are the most common serotypes prevalent in food isolates and human listeriosis cases (Jersek *et al*., 1999; Robinson *et al*., 2000; Wojciech *et al*., 2004; Liu, 2006; De Santis *et al*., 2007; Ryser & Marth, 2007; O'Connor *et al*., 2010). Serotype 4ab or more possibly 4b are the most common serotypes found in dairy products (Ryser & Marth, 1991; Eley, 1992; Robinson *et al*., 2000; Lunden *et al*., 2004; Ryser & Marth, 2007; Aurora *et al*., 2009). Various studies have specifically identified serotype 4b (Hui *et al*., 1994; Hamdi *et al*., 2007; Ryser & Marth, 2007; Bundrant *et al*., 2011) and 4ab (Holko *et al*., 2002) from *Listeria* in raw milk.

The genetic fingerprinting of the isolated *Listeria* was also determined to identify the most closely related strains. Since the observation from this study was that the isolated bacterial strains possess the same serotype it was necessary to characterise and separate them to strain level, thus ruling out the possibilies of one strain. With ERIC specific primers, amplification products per isolate were generated. Analysis of these genes revealed different profiles. When comparing the isolates for similarity by visual inspection of band patterns, three isolates showed similar DNA banding forms (refer to Figure. 4.5, page 71 lane 1,4 and 5), while two isolates (lanes 2 and 3) had different profiles.The typing pattern for these two strains do not match the three other isolates

although they did contain a few bands which demonstrate a low level of similarity. However, the similarity of the isolate in lane 3 with only a band difference to those in lane 1, 4 and 5 may suggest that they are clonally related. The similarity of the three identical isolates may be cause for alarm and suggests the possibility of an epidemic should such organisms contaminate the final products. Kells & Gilmour (2004) conducted a survey of milk processing plants and isolated *Listeria* in pasteurised milk from a 44% *Listeria* prevalence in raw milk. Alessandria *et al.* (2011) observed similarity of strains that were both isolated from the equipment and the final product when conducting molecular characterisation of *L. monocytogenes* in a dairy processing plant.

These results have demonstrated a good discriminatory capacity for identifying the serovars. Considering the significance of food as a vehicle for disesase transmission, detection of related strains can be used to dertemine sources of contamination in both the farm environment and manufacturing plants. Finally the obtained data indicated that comparison of these isolates can be used in epidemiological investigations and even to trace outbreak sources in a case where the products may be incriminated. These results also emphasise the need to implement programs that will employ molecular identification of pathogenic strains in the food industry along with measures that may guide infection control.

There may be regional differences in the prevalence of *L. monocytogenes* and this may warrant further study, since the area under study only covered a small region of the entire district.

CHAPTER V

RESULTS OF QUESTIONNAIRE

5.1 INTRODUCTION

Many people are usually affected with foodborne illness after consuming contaminated foods. However, it is difficult to determine the number of affected people if proper reporting is not carried out. People usually don't report because there is no place to report or sometimes symptoms are self resolving and therefore they don't consult for medial attention. Consequently, many cases are not diagnosed and if diagnosed, thorough investigations which will link the case of the illness to the source are usually not performed.

To determine the prevalence of consumption by the community, households were considered as a representative of the general population. A questionnaire was designed to collect demographic information, including perceptions related to foodborne illness experienced in the household as a result of milk and/or milk product consumption. Respondents resided in the Maseru City constituencies and sampling areas were randomly selected (refer to Chapter 2, Table 2.9). Only the household head or an adult representative (age>18) was considered suitable to respond on behalf of family members. For purposes of analysis, data were disaggregated to note differences in response and identify factors that may influence prevalence. Food poisoning symptoms that were experienced include amongst others nausea, vomiting, headache, diarrhoea, abdominal pain, fever and few other symptoms. To our knowledge this is the first documented study on foodborne illness with regard to dairy product consumption in Maseru, Lesotho.

5.2 CHARACTERISTICS OF RESPONDENTS

5.2.1 DEMOGRAPHIC CHARACTERISTICS

From 300 households 51 households were affected out of which 91 persons indicated symptoms alleged to be related to consumption of milk and/or milk products. Of the 91 respondents with alleged symptoms, 59 were females and 32 were males. None of the respondents indicated consumption of raw milk. The ages of the family members that indicated illness ranged from 7 to 53 years. Table 5.1 shows the background characteristics of complainants and the food items involved.

Table 5.1 Demographic characteristics of complainants and the food items involved

BACKROUND CHARACTERISTICS		**FOOD ITEMS**			
	Complainants (n=91)	**Sour milk (n=38)**	**Fresh milk (n=36)**	**Cheese (n=4)**	**Yoghurt (n=13)**
Gender					
Male	32	15	11	2	7
Female	59	23	25	2	6
Age (years)					
<10	5	0	4	0	1
11 - 20	12	4	4	0	4
21 - 30	21	8	11	3	0
31 - 40	26	13	12	0	0
41 - 50	22	11	5	1	5
51 - 60	5	2	0	0	3
Households					
Income category / **Affected houses**					
Low / 26	45	17	16	3	9
Middle / 17	25	12	10	1	2
High / 8	21	9	10	0	2

5.2.2 AGE DISTRIBUTION OF RESPONDENTS

The highest number of complaints was seen in age group 31 – 40 with 28 % (26/91), followed by age groups 41 - 50 with 24% (22/91) and 21 – 30 with 23 % (21/91). Results indicated a variation in the number of affected participants among the age groups. In high income households, a higher number of complaints were seen in the age group of 21 - 30 years while in the middle income households a high number was observed from age groups of 31 - 40 years and 41 - 50 years. The prevalence of complaints in low income households was significantly higher than in other households except in age group <10. However, the high income households had a higher number of complainants in the <10 age group than other households. In age group 11-20 years, the number of complainants was the same for both middle and high income households (Figure 5.1).

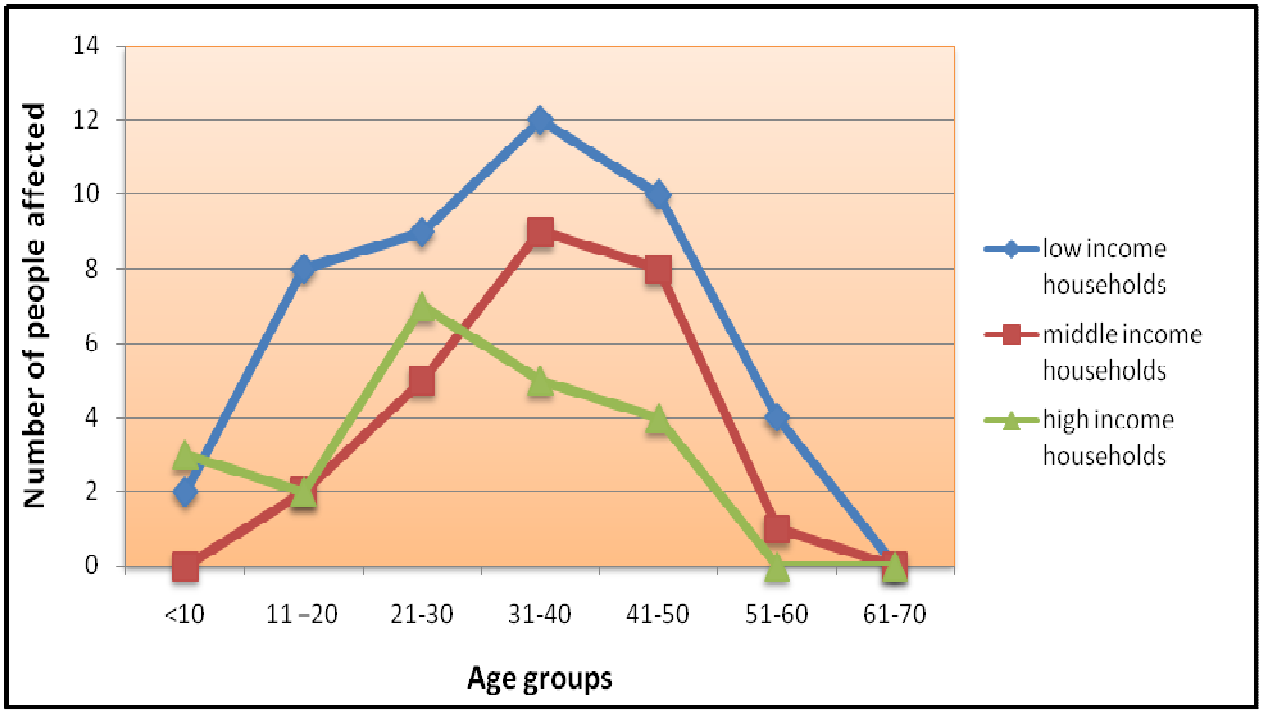

Figure 5.1: Age distribution of respondents

5.2.3 COMPLAINTS IN HOUSEHOLDS

In general, an average of 17% (51/300) of the households in MCC reported to have experienced foodborne illness due to dairy consumption. Out of 26 low income household, there were 45 complainants, while 17 Middle income households had 25 comlpainants and lastly 21 complainants from 8 high income households. Low income households appeared to have the highest percentage of complaints. Approximately 18.7% (26/139) of the sampled low income households complained of illness related to dairy consumption, followed by high income households with 16.7 % (8/48), while middle income households had the lowest percentage 15.1% (17/113) (Figure 5.2 and Figure 5.3).

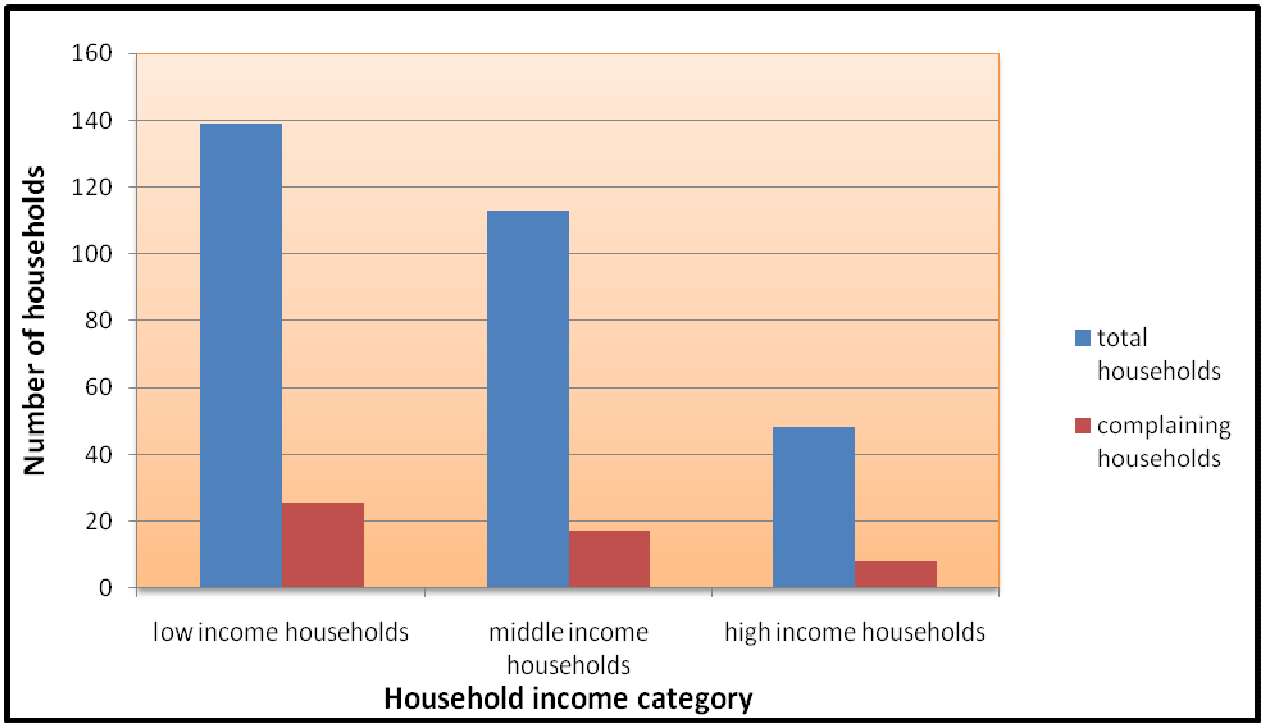

Figure 5.2: Complaints distribution of households

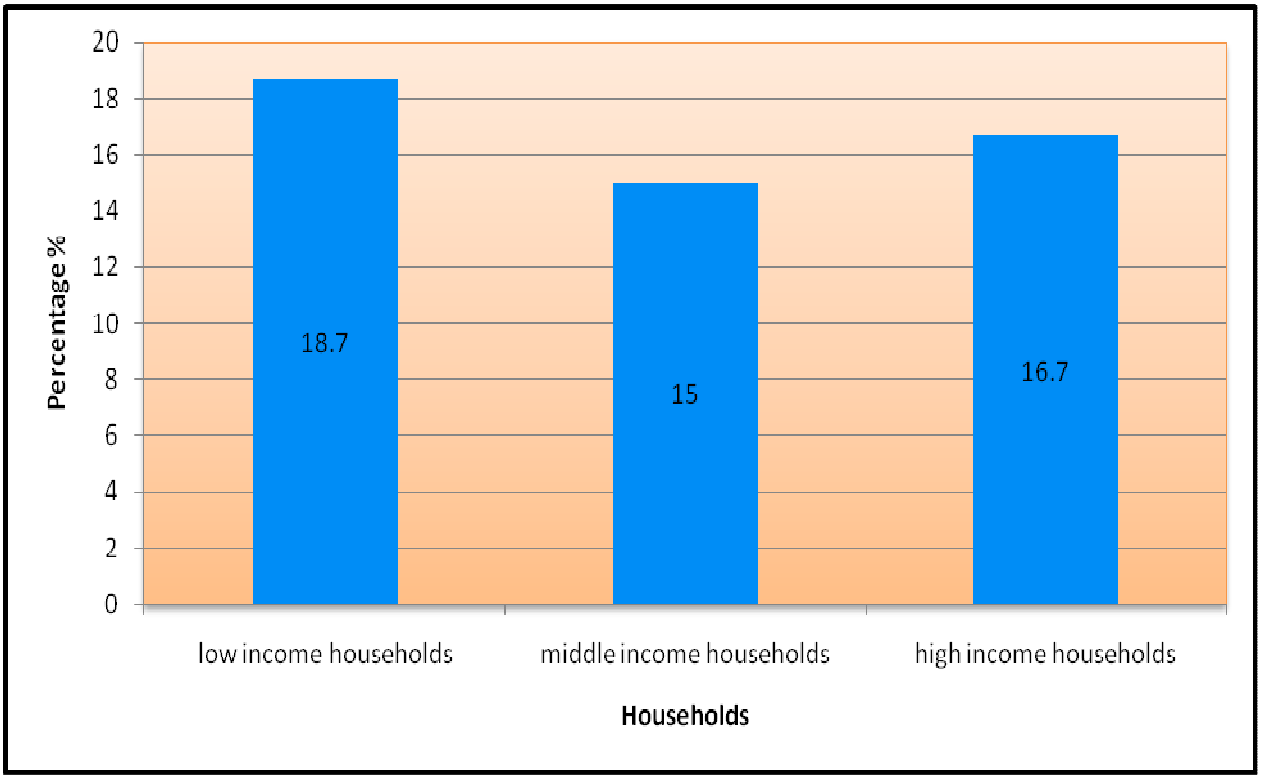

Figure 5.3: Percentage distribution of household complaints

Sour milk (38) and fresh pasteurised milk (36) were mostly implicated followed by fresh milk. All households indicated a high prevalence of complaints about sour and fresh milk than any other dairy product. However, low income households had higher prevalence of complaints than other households in all the four food items compared to other two households. A low prevalence of complaints was seen with Yoghurt in both the middle and high households except in the low households where it was higher, while that of cheese was the lowest in all households (Figure 5.4).

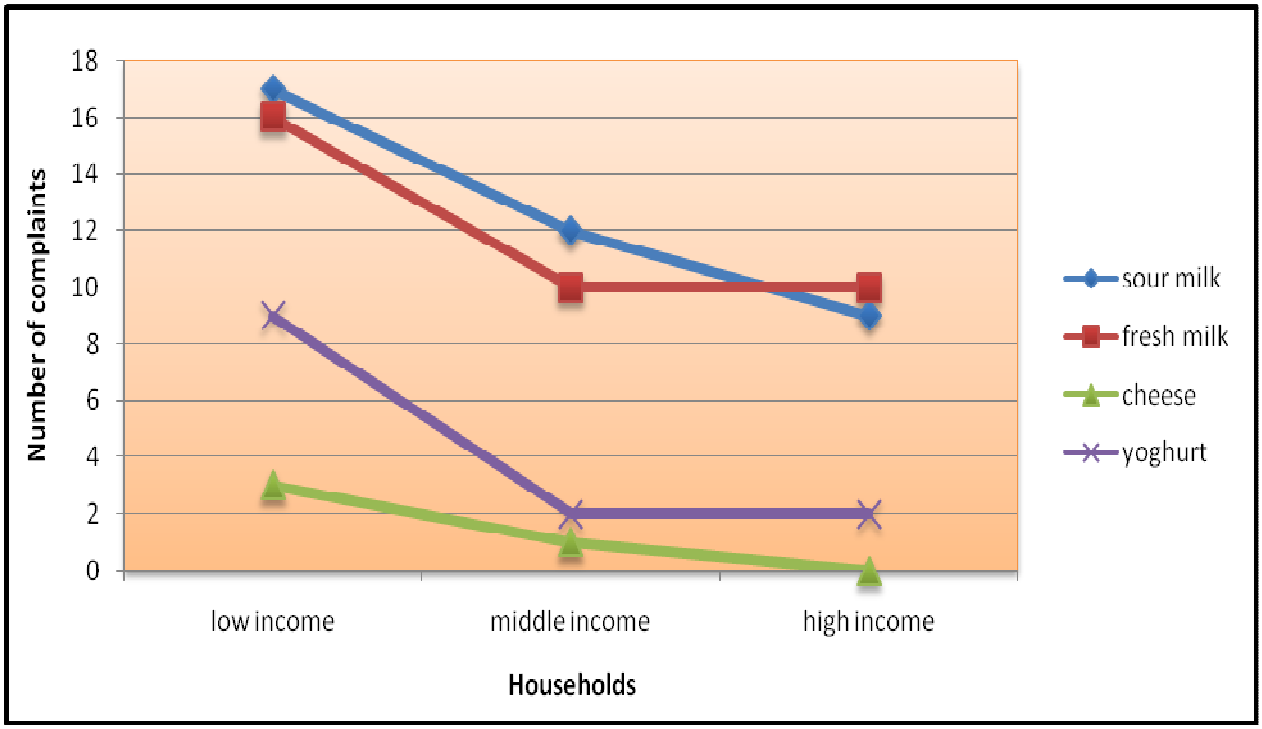

Figure 5.4: Household complaints per food item

5.2.4 SYMPTOMS

Abdominal pain, diarrhoea and nausea, were the most frequent indicated symptoms mainly associated with sour milk consumption followed by pasteurised milk consumption. Vomiting, headache, and fever were less frequently experienced, and the prevalence of the other mentioned symptoms were low. Complainants did not consult doctors as much as one could expect. Out of 91, only 30 (32.9%) opted for medical attention. Low income households seemed to be the ones with a high percentage 35.6% (16/45) of complaints who opted for medical attention, followed by middle income with 32% (8/25) and high income households being the last with a low incidence of 28.6 % (6/21). Figure 5.5 shows the distribution of symptoms experienced and Figure 5.6 shows the frequency of consultation among the complainants.

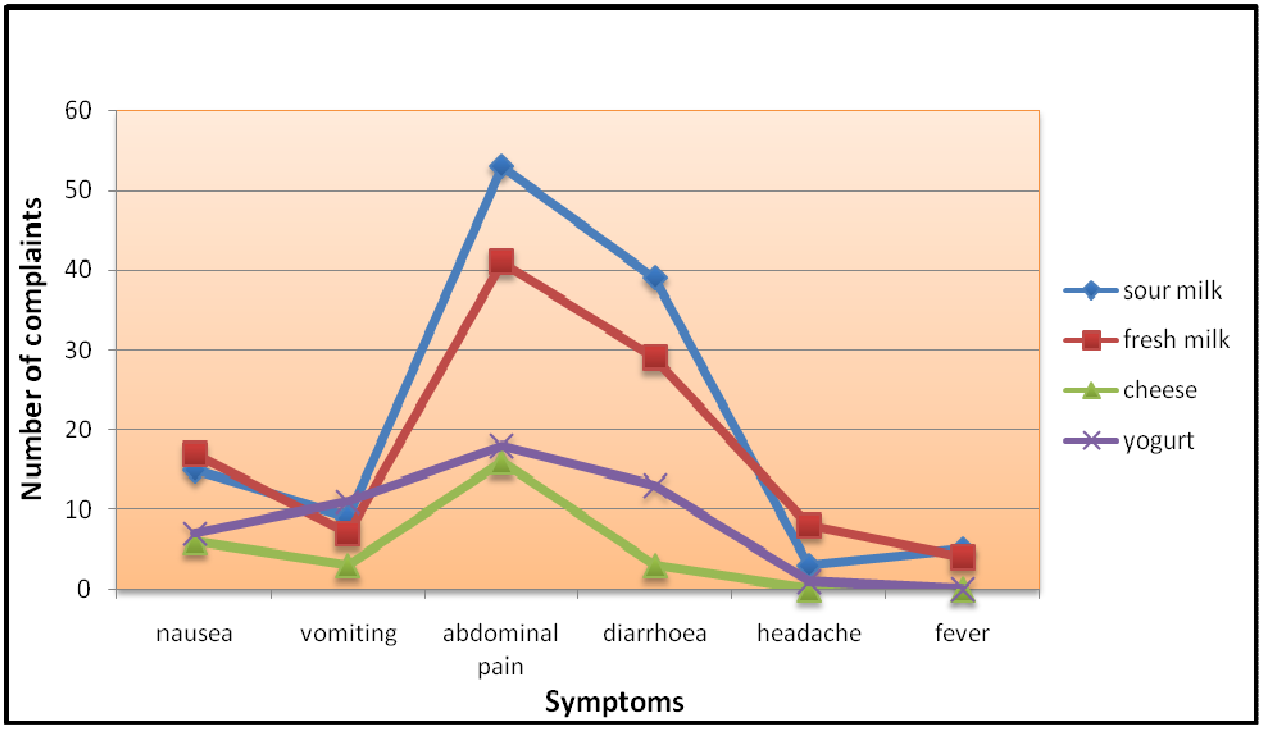

Figure 5.5: Distribution of clinical symptoms as experienced per food item

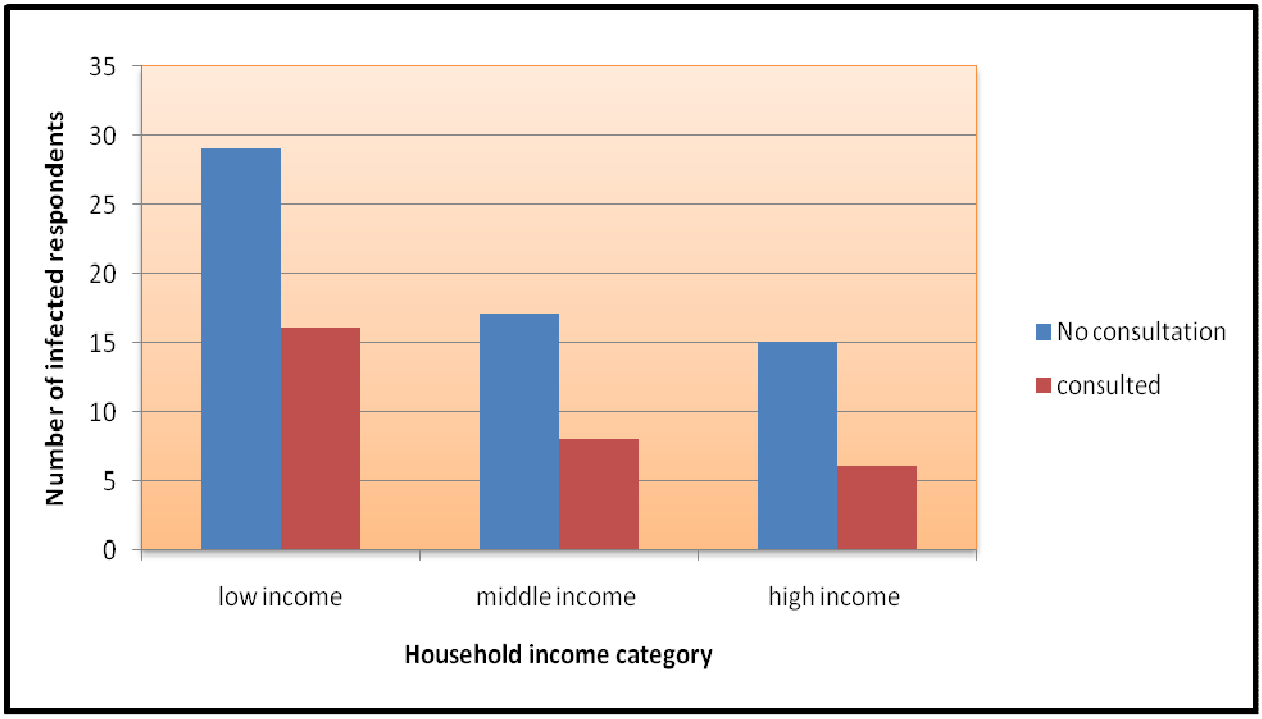

Figure 5.6: Distribution of infected persons according to treatment seeking behaviour by household income category

5.3 DISCUSSION

Complaints of persons in low income households do not differ from the middle income households in terms of age groups complaining of illness (Figure 5.1). The only significant difference was noted in age group 11 – 20 years where there were a higher number of complainants among the middle income households. The older age groups did not appear to be affected as would be expected. One reason could be that this average age within township households was not as high. The prevalence of symptoms was also low in the age groups <10 and 51- 60 and highest in age group 31- 40. No particular reason could be attributed to this finding. Both males and females were affected by the symptoms. However there seemed to be an evident variation between the two groups as 65% (59/91) females and 35% (32/91) males experienced symptoms. There was no significant variation in pattern of reporting by enumeration area. The low income households had a complaining percentage of 18.7% followed by the high income households with 16.7%, while the middle income had the lowest complaining prevalence of 15.1%. One would expect a higher percentage of complainants who consulted doctors, but this was not the case possibly due to the fact that the symptoms would resolve before the illness becomes severe. It is also important to note that 43% (22/51) of the respondents were unemployed and not at work as they were the ones at home during the time of survey.

Results indicate that 17% of the households indicated complaints related to dairy consumption. Consumption of sour milk and fresh milk had a significant effect on the community. About 81% of the illnesses (74/91) were attributed to fresh and sour milk consumption. Sour milk had the highest prevalence, followed by fresh milk. It is interesting to note that even though respondents not indicate any consumption of unpasteurised milk, the quality of the commercial milk available to the public is of questionable standards. The

quality of dairy products can be affected either by compromised processing standards, compromised storage facilities in the retails or compromised storage by the consumer. High counts of bacteria in milk can be associated with any milk organism and as such could be responsible for food-borne symptoms that may be experienced. However such high complaints did not apply to yoghurt and cheese products. The low prevalence observed for cheese and yoghurt in households amongst other reasons, may be due to the high acidic content (pH) of these fermented foods (Garrote *et al*., 2000) which has an effect on suppressing microbial numbers. Even though the consumption frequency was not indicated by the respondents, low prevalence could also be attributed to this factor. However, cold storage facility is equally important to maintain the quality of the product most importantly if the organisms present in food products are not psychrotrophs.

While most of the complainants associated a particular food product to a since incidence of alleged food poisoning, it was also observed that more than one incidence of alleged food poisoning by different dairy products was demonstrated by four complainants. The most prevalent symptoms were gastrointestinal, such as abdominal pain and diarrhoea. The presentation of these symptoms with such high prevalence is a point of concern. Vomiting, headache, and fever were less frequently experienced. Additional symptoms with a very low prevalence indicated by some respondents included weakness and loss of appetite.

The majority of people indicated symptoms that presented either the same day or the following day after consumption. And for some, the indicated symptoms lasted for only a day or two. Food poisoning organisms have incubation times that range from hours to days or even weeks (Robinson *et al*., 2000). In some cases of foodborne illness, some

days or weeks can pass before people experience symptoms of illness after they have ingested contaminated food. A challenge presented by this study is that firstly, the true numbers of people affected in the entire community may not be well reflected as a result of not being able to link symptoms to food that has been consumed days or weeks before. Secondly the incubation times stated by the respondents could not be attributed to any causative organism as they may have overlapping incubation times and similarity of presented symptoms. Lastly, the report is based on respondent's ussumptions of which the real causative food item may have been missed. This challenge may be addressed by proper public health laboratory investigations and surveillance.

In the majority of food-borne infections, the causative organism often remains unidentified due to less reporting of the cases and if they should be reported, only symptomatic treatment is given without identifying the causative organism. Food-borne outbreaks in healthy people are therefore not recorded as they are often mild and readily resolved (Eley, 1992). This study reported that only 32.9% of the affected population sought medical attention. The majority of complainants (66.1%) did not consider medical attention. One explanation for this could be the short period of illness indicated by most of the respondents. For those who consulted, 16.7% (5/30) reported that the stool sample was collected while 56.7% (17/30) said it was not collected and 26.6% (8/30) of them said they can't remember if it was collected. The low number of people who sought for medical consultation could be attributed to, among other reasons, challenge to immediate access to health centres and cost of medical care which could be a challenge to some sector of the community. Nevertheless, knowledge of the danger of foodborne illness is a critical factor in the decision to seek medical attention. Even though the information from the questionnaire was used to determine the household prevalence by households, age and

gender, the main aim was to determine consumption of bovine milk (unpasteurised or pasteurised) and its products. Future extension of the questionnaire will include other foods consumed, frequency of consumption of dairy products per day, interview with participants from both urban and rural areas to determine their knowledge of acceptable quality of dairy products and risks involved in consumption of dairy product of compromised quality.

5.4 CONCLUSION AND RECOMMENDATIONS

5.4.1 CONCLUSION

Successful participation in the collection of data showed that participants were eager to voice out their perceptions on dairy related illnesses. Even though other foods could be incriminated in foodborne illnesses, the main focus of this study was only on dairy products. All the interviewed households indicated consumption of milk and/or its products. These were dairy products that were purchased at retail shops. However, none of the participants indicated any consumption of raw milk. Results show that 83% (249/300) of the surveyed households consumed dairy products and did not indicate any problem due to their consumption. However, some households (17%) held different perceptions; dairy products consumed were allegedly implicated in food poisoning illnesses experienced. This study indicated that according to the respondent's perceptions, different symptoms were associated with milk and/or dairy products and the household prevalence of food-borne illness was found to be 17% in this area.

Storage condition is an important factor that determines the longevity of any perishable food product. Compromised quality of food could be caused by among others, lack of cold

storage facility, leading to a compromised state of the product even if it is still within its stated expiration date. Milk and dairy products, just like meats, are amongst the perishable foods (Arakawa *et al.,* 2008) that need uncompromised attention. Even though it is the responsibility of the manufacturer to assure that only those tested batches are in acceptable quality to be released for commercial purpose, it is also important for the consumer to be aware of factors that may compromise the quality of the foods. This is much so because the product may leave the manufacturer with acceptable standards. However, the standards may be compromised by the storage condition of either the seller or the consumer.

5.4.2 RECOMMENDATIONS

Ignorance concerning the importance of reporting was evident and this finding suggests that people are not aware of the importance of reporting. Health education is therefore required in order to educate the public. Consumer awareness will influence the response of authorities and the manufacturers.

Several notable challenges seen in this study should be addressed by the authorities in public health in order to determine true estimates of foodborne illness in Lesotho. Firstly, the people involved usually do not consult doctors. Secondly; doctors do not always perform thorough tests, as was discovered in this study. Finally, there is no pathology laboratory in Lesotho dedicated to public health for investigating foodborne illness. Determination of food poisoning prevalence is important for many reasons; firstly it serves as an indicator that will inform policy and strategies for prevention. Secondly the public health authorities can put in place structures that will monitor progress and control foodborne poisoning. Thirdly, indicators can be used to address knowledge and behavioural changes that promote reduction of foodborne outbreaks.

Food frequency data and collection of other factors pertaining to environmental food safety aspects were not within the scope of this study. It would be highly recommended that:

- Futher investigation be undertaken in order to inform policy and intervention strategies.
- The authorities are therefore required to strategise and implement the process for collecting, analysing and reporting of data. As part of that process, clinicians will also provide their input which includes submission of confirmed data and notification of identified cases to public health authorities.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSION

6.1 GENERAL DISCUSSION

The objective of this study was to determine the general microbial quality of milk in the Maseru area. Two hundred milk samples were analysed; 160 raw (unpasteurised) milk and 40 pasteurised milk samples. The use of microbial count tests revealed that raw milk samples did not conform to microbial standards and ranged from 54.4% with high aerobic plate count, 55.6% with high total coliform count to 21.9% with a high *E. coli* count. This may be attributed to negligence of proper sanitation practices as one of the leading factors that may have contributed to such high counts. This emphasises the need for maintenance of hygienic conditions in the milking environment. This finding concurs with observations made by Van Kessel *et al.* (2004) who found high levels of bacteria in bulk milk and emphasised the need for maintenance of hygienic conditions in both the milking and processing environment. The pasteurised milk samples also showed counts with unacceptably high levels of microorganisms. High aerobic plate count was seen in 67.5% of the pasteurised samples. However total coliform count was high in only 17.5% of the samples compared to 55.6% for raw milk. These could be attributed to ineffective pasteurisation processes or post processing contamination. In pasteurised milk there were more samples with a high aerobic count than in raw milk. However, *E. coli* was not isolated in pasteurised milk samples. It can therefore be concluded that based on the microbial analysis of milk samples bought from local shops, some milk sold to the public did not conform to the national legislation concerning the presence of bacteria in pasteurised milk.

Results also indicated that some of the raw milk available to the community was contaminated with *Listeria* but not observed in pasteurised milk samples. The prevalence of *Listeria* was found to be 3.75%. *Listeria* species were found in six samples tested. Five samples were contaminated with *L. monocytogenes* and one sample with *L. innocua*. The isolates belonged to serotypes in lineages I or III. Despite having the same serotype, Characterisation of isolates revealed three different genetic fingerprinting profiles. Three isolates had similar fingerprinting patterns while two had different patterns. Such information is beneficial for implementation of measures for control and prevention of outbreaks and for epidemiological surveys. The presence of *Listeria* in food is an issue that raises much concern as this organism is responsible for cases of listeriosis. Prevention of listeriosis has to be of major importance as it poses a risk to especially pregnant women and immune-compromised individuals. If the prevalence of *L. monocytogenes* is successfully reduced in dairy products, the risk of acquiring listeriosis from the products will also be reduced. Although the Maseru Dairy utilises the pasteurisation process as it is considered an efficient and safe process for elimination of *L. monocytogenes,* it is important to note that the product may become contaminated after heat treatment by the dairy environment. Numerous studies documented the presence of *Listeria* within the dairy processing plants (Hayes *et al*., 1986; Van Kessel *et al*., 2004; De Santis *et al.,* 2007; Alessandria *et al.,* 2011). Thus, pasteurisation of raw milk alone does not eliminate the risk of *L. monocytogenes* in milk

Although there was no indicated prevalence of raw (unpasteurised) milk consumption from the community, participants indicated symptoms alleged to consumption of pasteurised milk and/or milk products. A high prevalence of symptoms was observed with both sour and fresh milk consumption but it was somewhat lower in cheese and yughurt

consumption. The number of respondents with symptoms was variable with different age groups, with the middle age group being the most affected. There was no significant difference in terms of complaints among different households. Prevention of foodborne illness is a universal target. Although more respondents indicated no illness to be associated with dairy consumption, negative allegations appear to be prevalent, presenting a challenge for public health intervention. Few complainants opted for medical consultation indicating a lack of knowledge concerning the importace of reporting foodborne illnesses.

6.2 GENERAL RECOMMENDATIONS

Farmers in Maseru can also play a crucial role in prevention of listeriosis. The degree of cleanliness of the farm environment and milking system will influence the milk bacteria as much as any other. In this case measures should be applied to minimise contamination of the raw product. Since such contaminants can influence milk counts, proper teat preparation before milking is crucial. Milk residue left on equipment contact surfaces supports the growth of a variety of microorganisms and thorough cleaning and sanitation of equipment should also be emphasised (Ryser & Marth, 2007).

- Dairy products are sensitive and perishable items that cannot be dealt with without proper hygiene.The Food Safety Programme under the Ministry of Health in Maseru has compiled food safety manual that should be adapted by food handlers and producers in order to demonstrate the required good manufacturing practices.
- In infected animals, early treatment with antibiotics is highly recommended. However animals under treatment may be excluded from milking according to the

specified recommendations. Methods of prevention such as vaccination against listeriosis are still under investigation.

- Measure that should be employed to ensure adequate food safety practices. Information about the risks involved in consuming unpasteurised milk or milk products should be provided continuously to vulnerable groups of the population, pregnant women, the elderly and the immunocompromised (Eley, 1992). Teachings concerning food safety could be discussed at health centres and health warnings should be issued in relation to high risk foods. In cases where raw milk sales are permitted directly from dairy farms, communities need to be educated that the milk may contain organisms that are harmful to health, and therefore has to be boiled prior to consumption.

Food safety is an issue that cannot be avoided and to provide a safe product to the public, it is essential to improve hygiene standards in dairy industries. It is difficult to say if these alleged food poisoning symptoms reported in this study were due to food safety challenges at production level as many reports indicate that various aspects of food safety can be addressed at production level. However, the authorities can put proposals to develop strategic approaches to hazard analysis and controls in foods. Since analysis of the final product for pathogens or indicators of spoilage organisms serves to verify that good manufacturing practices are carried out, food industries need access to rapid, reliable and sensitive methods of detecting bacteria (Ryser & Marth, 2007). Even though rapid methods are valuable tools, they may not replace standard culture methods.

- Testing for the microbial quality of milk can serve to help a dairy producer to identify inefficiencies in the production of milk and will also help in monitoring the quality of

pasteurised milk. At the same frequent inspection of equipment and operations is necessary and therefore highly recommended to the producers at Maseru if safe products of acceptable quality is their concern. This will assure that final products on the shelf meet the public's expectations for a safe and nutritious food.

Health and safety authorities in Maseru have an important role in checking the hygiene in dairy plants and to ensure that good manufacturing practices such as HACCP approach are in place and are strictly adhered to by food production companies. It is endeavoured that the recommendations and the use of these findings will help local dairy farmers produce high quality milk.

It may be worthwhile and interesting to expand this study and have it performed in a larger area and over a longer period of time to determine possible geographical variation and seasonality with regard to *Listeria* prevalence. This will also help to monitor the prevalence of *Listeria* especially in those places where it was identified in this study. Expansion of the study area will also allow analysis of a larger number of isolates which can be differentiated further through typing to establish an epidemiological profile. Further studies are also required to investigate the proportion of illness mainly due to food-borne transmission for specific pathogens. With such, estimates of the incidence of listeriosis and other food-borne illnesses can be well established at national level. This will provide a comprehensive study that will assist and enable the authorities to identify and prioritise areas that need critical attention. In view of epidemiology, matching of implicated food and clinical isolates during an outbreak may help in recognising and containing the source of food-borne infection.

APPENDIX

Prevalence of milk and/or milk product consumption Questionnaire

Demographic details

1. Location: Maseru City Council
2. Constituency:_____
3. Enumeration Area:_____
4. Name of Household: _____
5. Gender: _____
6. Age: _____

Consumption Details

7. Type of milk consumed most of the time

Raw milk	1 (YES)	2 (NO)
Pasteurised milk	1 (YES)	2 (NO)

8. How often does members of your household take milk?

1 (everyday)	2 (two times a week)	3 (more than two times a week)

9. Have any members of the household experienced any problem resulting from consumption of **milk**?

1 (YES)	2 (NO)

If NO, skip to Question 19. If YES, proceed to Question 13.

10. What kind of illness? - Tick the symptoms experienced

Nausea	1 (YES)	2 (NO)
Vomiting	1 (YES)	2 (NO)
Abdominal pain	1 (YES)	2 (NO)
Diarrhoea	1 (YES)	2 (NO)
Headache	1 (YES)	2 (NO)
Fever	1 (YES)	2 (NO)

15. How long after consumption did the symptoms start ?

1 (same day)	2 (one day)	3 (more than one day)

16. How long did the symptoms last?

1(one day)	2 (two days)	3 (more than two days)

17. Did you see a doctor about this illness?

1 (YES)	2 (NO)

If *YES*, was a stool sample obtained?

1 (YES)	2 (NO)

18. How many members of the household had the same problem?

No.	Age	gender
1		
2		
3		
4		
5		
6		
7		

19. Does the household consume products other than milk?

1 (YES)	2 (NO)

If NO, **END**
If YES, **proceed** to Question 20

20. Which milk product/s do they take?

1 - Sour milk	2 - Cheese	3 - Yoghurt

21. Has any member of the household experienced any problem resulting from consumption of milk products**?**

1 (YES)	2 (NO)

If NO, **END**
If YES, **proceed** to Question 22

22. What kind of illness? - Tick the symptoms experienced

Nausea	1 (YES)	2 (NO)
Vomiting	1 (YES)	2 (NO)
Abdominal pain	1 (YES)	2 (NO)
Diarrhoea	1 (YES)	2 (NO)
Headache	1 (YES)	2 (NO)
Fever	1 (YES)	2 (NO)

23. How long after consumption did the symptoms start?

1 (same day)	2 (one day)	3 (more than one day)

24. How long did the symptoms last?

1(one day)	2 (two days)	3 (more than two days)

25. Did you see a doctor about this illness?

1 (YES)	2 (NO)

If *YES*, was a stool sample obtained?

1 (YES)	2 (NO)

26. How many members of the household had the same problem?

No.	Age	gender
1		
2		
3		
4		
5		
6		
7		

27. According to your perception, which milk product/s was/were responsible for the illness?

1 - Sour milk	2 - Cheese	3 - Yoghurt

Any other information

_____
_____
_____
_____
_____
_____
_____

THANK YOU FOR YOUR TIME

Appendix 1: Questionnaire

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