Shigatoxin producing *Escherichia coli* O157 and non-O157 serotypes in producer-distributor bulk milk

By

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DECLARATION

I, Victor Ntuli declare that the thesis, which I hereby submit for the degree Ph.D. Food Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

June, 2017

ABSTRACT

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Several recent large outbreaks of gastrointestinal diseases have highlighted the threat posed by morbidity and mortality associated with shigatoxin-producing *Escherichia coli* (STEC). Furthermore, the treatment of STEC infections is now threatened by the emergence of antibiotic resistance which is an alarming health concern in the world of medicine. The most implicated STEC in foodborne disease outbreaks across the globe is O157 serotype, although some emerging STEC non-O157 serotypes are increasingly becoming recognised as foodborne pathogens of important public health concern. This study was undertaken to characterise bacterial species in raw and pasteurised producer-distributor bulk milk (PDBM) with specific emphasis on *E. coli* and other Enterobacteriaceae. *E. coli* was further investigated for the prevalence and distribution of virulence factors (*stx* 1, *stx* 2 and *hly*A), serotypes and antibiotic resistance patterns, which also

included extended-spectrum β-lactamase (ESBL) producing capacity. Subsequently, the study further estimated the haemolytic uraemic syndrome (HUS) risk associated with the consumption of STEC contaminated PDBM and also estimated the resulting burden of illness that may be associated with the consumption of such milk in South Africa (SA).

A total of 258 PDBM samples were collected, using convenience sampling, from outlets (purchase points) in eight different geographical provinces in SA. Isolation, detection and enumeration of total aerobic bacteria, coliforms and *E. coli* were carried out using 3M *E. coli* /coliform petrifilm plates. Matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) was used for the rapid identification of the bacterial isolates. The identification of *E. coli* was confirmed using PCR of the *uidA* gene. Further characterisation of *E. coli* into serogroups, identification of virulence factors and antibiotic resistance profiles were then performed. *E. coli* O157 was characterised using selective media and confirmed using mismatch amplification mutation assay (MAMA)-multiplex PCR. Identification of *E. coli* -serogroups was carried out by the restriction of amplified O-antigen gene cluster (rfb-RFLP), coupled with serum agglutination assay. Virulence factors (*stx* 1, *stx* 2 *and hlyA*) were determined using both phenotypic and genotypic characterisation. Antimicrobial agent susceptibility tests and detection of extended spectrum beta-lactamase (ESBL) producing capacity of the *E. coli* isolates were performed using phenotypic characterisation. Finally, a quantitative risk assessment for STEC in PDBM was also conducted.

More than 60% of the PDBM samples were found not to be fit for human consumption on the basis of the minimum standards prescribed in the Foodstuffs, Cosmetics and Disinfectants Act (Act 54 of 1972). Raw and pasteurised PDBM was contaminated with a wide diversity of Enterobacteriaceae species, which included spoilage microbiota.

Multidrug resistant *E. coli* strains, as well as strains potentially capable of hydrolysing the 4th generation cephalosporins through producing extended spectrum β-lactamases were isolated from PDBM and this is a cause for concern as these strains are a potential source of antimicrobial resistant foodborne pathogens to humans through the food chain. Genotypic and phenotypic analysis of the *E. coli* confirmed that these strains are diverse, carry a variety of STEC virulence factor encoding genes associated with human diarrhoeal diseases and belong to O157 and non-O157 seropathotypes. However, the non-O157 STEC seropathotypes detected were not the serotypes implicated in foodborne illnesses in literature surveyed, mostly from Europe, Japan, Canada and the USA. Given that STEC contaminated PDBM may pose a significant public health threat to consumers, a quantitative microbial risk assessment was, therefore, performed. The assessment revealed that there was a higher risk of HUS cases per year for consumers of raw than pasteurised PDBM and also in age groups below 5 years. The risk is dependent on the variability surrounding the risk profile of the milk and is explicitly influenced by consumer behaviour.

The study, therefore, recommends strict enforcement of and adherence to national standards, which regulate food hygiene in the dairy industry, especially for producers and suppliers of PDBM. Furthermore, the training on dairy technology and safety for producers and suppliers of PDBM by the relevant authorities need to be strengthened to improve public health and safety. The raising of awareness of the health risks associated with the consumption of raw milk for, particularly, consumers of raw PDBM, also needs to be scaled-up for them to make informed decisions when buying and consuming milk. The awareness will indirectly encourage consumers to buy certified raw milk.

DEDICATION

To God the Almighty,

my wife, Maleseli Ntuli and my kids Gina, Leseli and Naleli

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CHAPTER ONE

GENERAL INTRODUCTION

The South African (SA) dairy industry is characterised by the primary sector (dealing with milk production at farms) and the secondary sector (consisting of milk processors). Within the secondary sector, a few large processors operate nationally in more than one region while smaller processors operate in specific areas, and a number of milk producers sell their own produce to retailers and consumers, known as producer-distributors (PDs) (MPO, 2016). Milk sold by PDs, also known as producer-distributor bulk milk (PDBM), is typically (i) raw milk for human consumption, (ii) raw milk received with the intention of processing and selling as pasteurised milk, and (iii) pasteurised milk received to be sold, that has been pasteurised elsewhere at an approved facility. Surveys carried out at national, provincial and district levels on milk produced and supplied by PDs have highlighted microbiological inadequacy, which can pose a public health risk (Lues et al., 2003; O'Ferrall-Berndt, 2003; Cawe, 2006; Lues et al., 2010; Caine et al., 2014).

In 2013, a survey conducted on PDBM by a non-Governmental Organisation, the Dairy Standard Agency (DSA), in collaboration with Environmental Health Officers (EHOs) (in the different municipalities across SA) reported counts of *E. coli* which were more than stipulated in the Foodstuff, Cosmetics and Disinfectants Act (SA, 2001 Act (54), (1972)).

E. coli is commensal although a few strains have a potential of causing fatal human infections (Kaper et al., 2004). Pathogenic *E. coli* have been linked to foodborne illnesses across the globe and are classified into pathotypes, i.e. a group of strains that cause a common disease using a common and remarkable assortment of virulence factors (Nataro and Kaper, 1998). The most implicated *E. coli* pathotypes in foodborne outbreaks, including milk, are the shigatoxin producing (STEC) pathotypes, which are also known as enterohaemorrhagic *E. coli* (EHEC), particularly O157 (Rangel et al., 2005; Control and Prevention, 2007). However, many other non-O157 STEC serogroups are gaining importance as foodborne pathogens. *E. coli* O26, O91, O103, O111, O118,

O145, O104 and O166 are the non-O157 serogroups that most often cause illnesses in the USA, Europe, Japan and Canada (Constantiniu, 2002; Mathusa et al., 2010; EFSA, 2015). STEC illnesses are characterised by severe crampy abdominal pain, initial watery diarrhea, followed by grossly bloody diarrhea, and little or no fever, typical of hemorrhagic colitis (HC) (Nataro and Kaper, 1998). STEC can also cause life threatening haemolytic uremic syndrome (HUS), which later develops into acute renal failure (thrombocytopenia, and microangiopathic haemolytic anaemia) (Gyles, 2007). Although sporadic cases of infection by STEC greatly outnumber outbreak cases, outbreaks related to STEC O157 and non-O157 strains associated with milk were confirmed in continents such as the USA, Europe and Asia (CDC, 2008; EFSA, 2015).

The presumptive route of STEC in milk is through faecal contamination and also direct shedding from an infected udder (Farrokh et al., 2013). STEC infections are becoming cumbersome as a result of emerging antibiotic resistant strains (Tadesse, 2012). The spread of resistant *E. coli* has threatened treatment and is becoming a global public health concern (CDC, 2013b). Selective pressure exerted by antimicrobials used as growth promoters in animal feeds and for treatment has been argued as the major driving force behind the emergence and spread of drug-resistant traits among pathogenic and commensal bacteria. This notion has been supported by Khachatourians (1998), who report the use of antibiotics at therapeutic and subtherapeutic (in feed) levels and the occurrence of antibiotic-resistant *E. coli* O157:H7 isolates. *E. coli* is reported among the top six drug resistant bacteria which need novel therapies (Shah et al., 2004).

Owing to the lack of epidemiological data, the burden of pathogenic *E. coli* linked to the consumption of PDBM in SA has not been assessed. To gain an insight on the accurate estimates of the actual risk posed by the consumption of PDBM contaminated by pathogenic *E. coli*, a quantitative microbial risk assessment modelling is the best practice to evaluate food health risks

and control (CAC, 1998; FAO/WHO, 2003). Several risk assessment studies have been conducted in the USA, Europe and Africa in an attempt to quantify disease cases and burden as a result of milkborne pathogens (Grace et al., 2008; Clough et al., 2009; Giacometti et al., 2015).

Therefore, this study characterised *E. coli*, other Enterobacteriaceae and other bacterial species in raw and pasteurised PDBM in SA. The study also estimated the HUS risk associated with the consumption of STEC contaminated PDBM and estimated the resulting burden of illness that may be associated with the consumption of such milk in SA.

CHAPTER TWO

LITERATURE REVIEW

2.1. INTRODUCTION

The purpose of this review was to assess the importance of milk, including dairy products and the associated safety challenges resulting from contaminated milk. Foodborne outbreaks linked to the consumption of contaminated PDBM were discussed. The review was also aimed at understanding the pathogenicity, virulence factors, antibiotic resistance and the diversity of STEC O157 and non-O157 serotypes in food, including milk. The tool used to evaluate the risk posed by pathogens and the resulting burden of illness in contaminated food was also reviewed.

2.2. MILK AND DAIRY PRODUCTION

Milk is the secretion of the mammae of the female mammal intended for nourishment of their young. It is obtained from dairy animals, with neither addition to it nor extraction from it, and is intended for further processing or consumption as liquid milk (Codex Alimentarius, 1999). According to the European Union (EU) legislation, raw milk is defined as milk produced by the secretion of the mammary gland of farmed animals that has not been heated to more than 40 °C or undergone any treatment that has an equivalent effect (Regulation (EC) No 853/2004). Humans have consumed milk from several farmed animals (cows, buffalos, goats, sheep, camels and horses) for centuries and it serves as a good source of carbohydrates (lactose), animal proteins, fats, vitamins and minerals (Haug et al., 2007). Cow or bovine milk is by far the most consumed (Haenlein, 2004; Haug et al., 2007). Therefore, in this study, the term ‘milk’ refers to cow/bovine milk. Milk is produced and marketed raw or heat-treated (pasteurised, sterilised or ultra-heat treated).

The Food and Agricultural Organisation (FAO) (2016) reports that the world milk production grew by 1.6% in 2016 and reached 816 million tonnes per annum. Currently, the highest milk producing

countries in the world are India, the USA, Pakistan, Brazil and Germany, whereas, in Africa, the top five milk producing countries are Sudan, Egypt, Kenya, South Africa (SA) and Algeria (FAOSTAT, 2006). However, the FAO (2016) projects that milk production is set to expand in Europe, Asia and the USA but will remain stagnant in Africa. The growth in dairy consumption is driven by higher per-capita consumption and population growth (Coetzee, 2014). According to the FAO (2016), more than 6.5 billion people consume milk and dairy products and the majority of these are in developing countries. Figure 2.1 depicts the trends in global milk and dairy consumption and the world population. The International Farm Comparison Network (IFCN) recently reported that, in the next three years, the global dairy demand will grow by 20 million tonnes a year, of which 8 million is as a result of population growth and 12 million as a result of higher per capita consumption (IFCN, 2017). The annual global increase in dairy demand from 1997-2017 is presented in Figure 2.2.

Milk is highly perishable and because of this nature, it must often be processed into a more stable form to allow a longer shelflife and improve its microbiological safety, or be transported quickly to a place where it can be refrigerated to prevent bacterial growth. Owing to the aforementioned important nutritive properties of milk, many products (with an extended shelflife) are processed from milk, offering a wide variety of dairy products such as standardised milk, pasteurised milk, cream, skim milk, butter milk, cheese, butter, evaporated or condensed milk, milk powder, casein and other products (Kamana, 2015). Milk and dairy products are income-generating and as of 2012, the top five countries in the world made an average turnover of 15 billion USD each, in the industry (Coetzee, 2014).

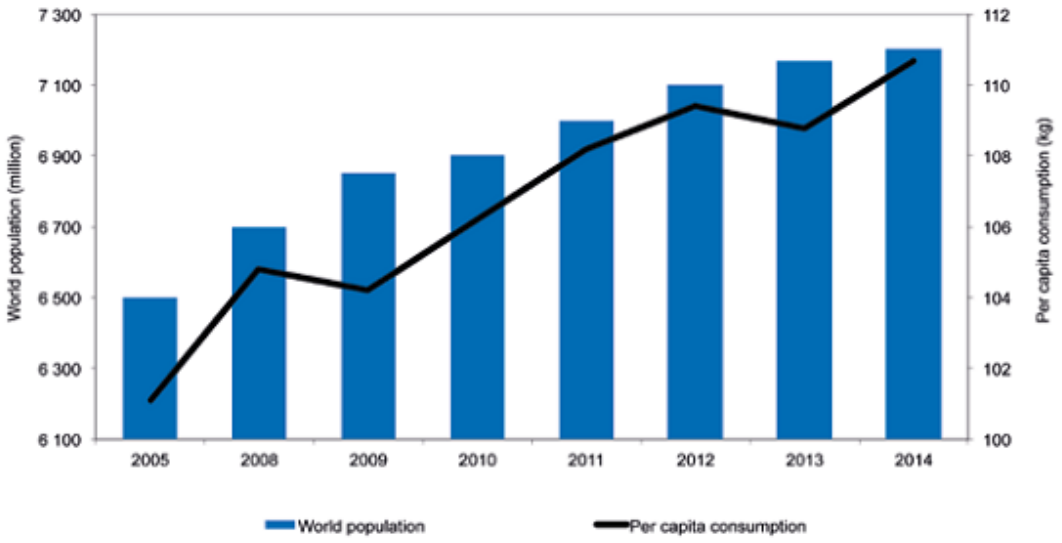

Figure 2.1: World population and per capita consumption of dairy products, 2005, 2008 – 2014 (IDF Bull. 481/2015).

The dairy industry has several characteristics that set it apart from other areas of agriculture and these are diverse and complex, involving everything from production (farming), processing, distribution, trade (multinational companies) and regulation (Hueston, 2012). Different milk production and marketing practices have evolved over time, sometimes bringing with them unforeseen and adverse quality consequences to milk itself. The scale and complexities of today's dairy industry contribute to food safety challenges and the likelihood and magnitude of foodborne illnesses (Ercsey-Ravasz et al., 2012). As a result, there is a need to enforce strict and comprehensive quality regulation in the dairy industry, especially in developing countries, to protect public health.

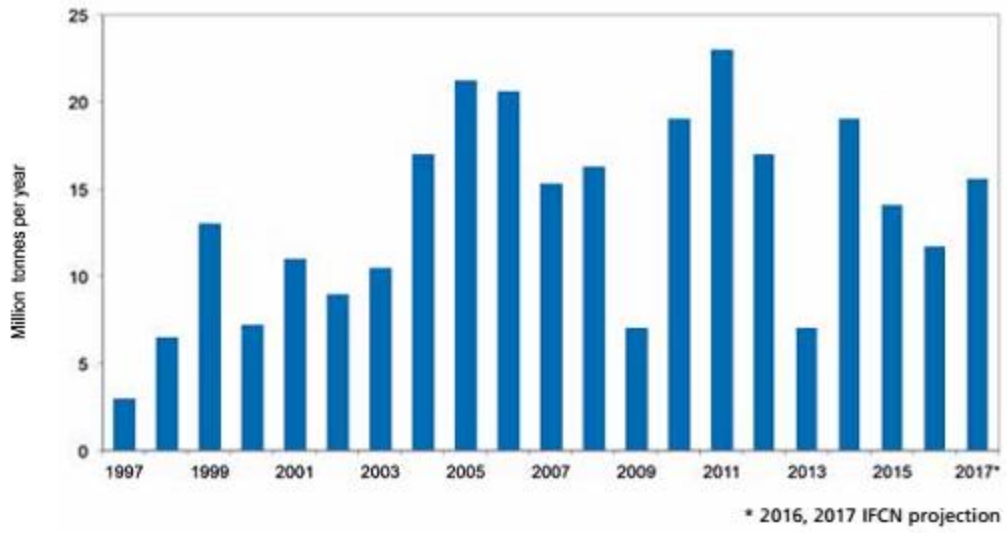

Figure 2.2: Annual global increase in dairy demand, 1997 – 2017 (IFCN 2016).

2.3. MILK PRODUCTION IN SOUTH AFRICA

The SA dairy industry is one of the largest in Africa, contributing about 0.54% of the world milk production (FAOSTAT, 2006; DAFF, 2014). It is the fourth largest agricultural industry in the country, producing an average of 3 million tonnes of milk per-year and representing 5.6% of the gross value of all agricultural production (Mkhabela and Mndeme, 2010; MPO, 2016). The number of milk producers has decreased by more than 50% (Table 2.1) from 3 665 in January 2008 to 1 683 in January 2016 (MPO, 2016). Within the stated period, Free State followed by Western Cape were the leading milk producing provinces in the country while Northern Cape and Limpopo provinces were the least. The biggest percentage decrease in producer numbers occurred in the Free State (69,5%). The Department of Agriculture, Forestry and Fisheries (DAFF) (2014) reports that coastal areas were more suitable for milk production because of mild temperatures, as well as good rainfall that ensures good quality natural and artificial pastures. The inland production areas are generally climatically less favourable for milk production (DAFF, 2014).

The Milk Producers Organisation (MPO) carried out a survey in 2014 and documented the milk production per-unit area per-district (Figure 2.3). Milk in SA is produced through three farming systems, namely the open-grazing, semi-grazing and zero-grazing systems, although the open-based, which favours coastal areas, is the most common (MPO, 2016).

Table 2.1: Number of milk producers per province in South Africa, 2008-2016 (Milk Producers Organisation, 2016).

Province	**Year**						
	2008	**2009**	**2011**	**2012**	**2014**	**2015**	**2016**
Western Cape	815	795	683	647	529	533	502
Eastern Cape	407	387	314	283	264	262	251
Northern Cape	34	37	28	21	25	14	14
KwaZulu-Natal	373	373	323	322	281	267	253
Free State	919	884	601	535	389	328	280
North West	549	540	386	352	233	222	181
Gauteng	228	217	127	126	109	100	97
Mpumalanga	302	286	201	164	117	94	93
Limpopo	38	32	23	24	14	14	12
Total	**3665**	**3551**	**2686**	**2474**	**1961**	**1834**	**1683**

*The statistics were recorded in January of each year.

Cattle are grazed mainly on pasture in summer while supplementary feed, such as baled hay and grass silage are used in winter. However, it is a common practice in most provinces in SA for farmers to store plastic encased balayage. Archer (2014) observed that this is an anaerobic environment in which spore-forming, heat-stable bacteria can grow and if such bacteria enter milk, they will be transported to a processing plant, gain entry and survive heat treatment. Once bacteria have entered the milk chain, they are able to colonise within fouling films that can build up on milk contact surfaces and ultimately contaminate the product (Archer, 2014).

The secondary dairy industry in SA consists of a few large processors operating nationally in more than one region, smaller processors who operate in specific areas and a number of milk producers

who sell their own produce to retailers and consumers, known as producer-distributors (PDs) (MPO, 2016). Large and some small processors receive the bulk of the milk (98%) from a more formal sector which often meets the strict and comprehensive dairy standards in SA (O'Ferrall-Berndt, 2003). However, following the deregulation of the SA dairy industry in the early 1990s (O'Ferrall-Berndt, 2003), emerging milk processors, who produce and sell their milk (2%) directly to the public (known as PDs), have become common (O'Ferrall-Berndt, 2003; Agenbag, 2008; MPO, 2016). Although this initiative can improve the incomes and livelihoods of farmers, quality and safety of the product are of paramount importance (DAFF, 2012).

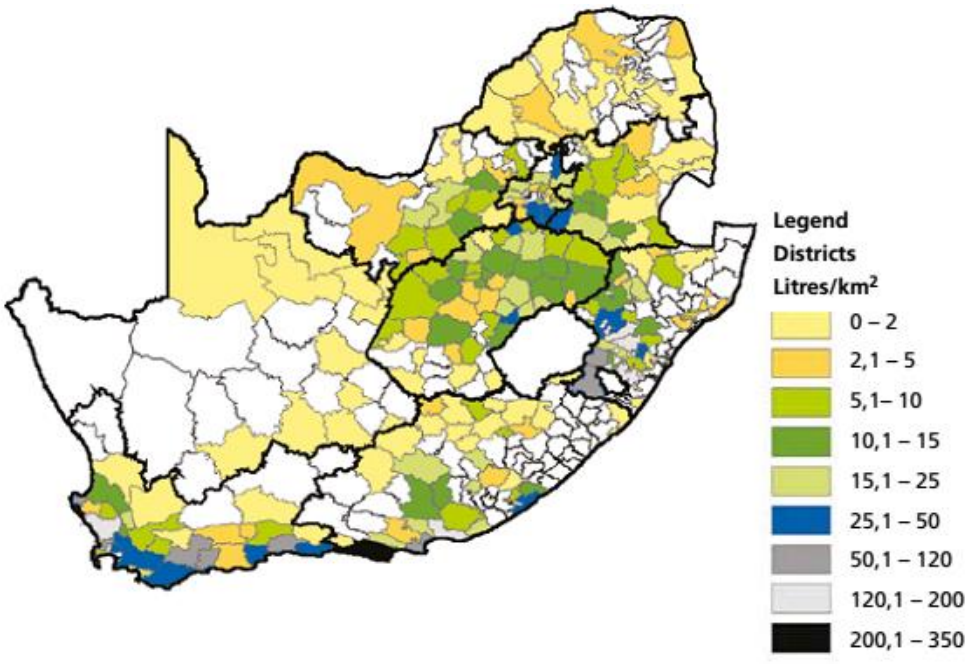

Figure 2.3: Milk production density (litres/km²) per district in South Africa in 2014 (MPO, 2016).

Albeit, the extent of microbiological quality of milk from PDs is not clear, several researchers on milk quality and safety in SA continue to highlight microbiological inadequacies (according to Foodstuffs, Cosmetics and Disinfectants Act (54), 1972) in the quality of this milk (Lues et al., 2003; O'Ferrall-Berndt, 2003; Agenbag, 2008; Lues et al., 2010; MPO, 2016). Current statistics

have revealed that, between September 2008 and August 2016, the number of PDs decreased by 37.6% (MPO, 2016).

2.3.1. South African milk value chain

The SA milk value chain is characterised by the interconnection between the formal and informal market (Agenbag, 2008; DAFF, 2014). Figure 2.4 presents the structure of the SA milk and dairy value chain. The production of milk and dairy products in SA follows several stages and generally starts with animal feed production, followed by raw milk production at the farm, and further processing, either at a dairy company or at the farm itself. Spoilage and pathogenic organisms may enter at any stage along the dairy supply chain. *E. coli* pathotypes, particularly STEC O157, are one of the most commonly isolated pathogens in milk. Potential sources of STEC in milk can be water used for cleaning milking equipment or faecal contamination along the dairy value chain. Several studies in SA have isolated and detected STEC in water and cattle faeces (Ateba and Bezuidenhout, 2008; Aijuka et al., 2014; Iweriebor et al., 2015; Msolo, 2016).

2.3.1.1. Milk from the formal value chain

The formal value chain of milk in SA typically consists of commercial dairy farms, although some small and medium milk producers also exist (Figure 2.4). Recipients of milk from the formal value chain are national distributors such as Clover, Dairybelle and Parmalat, who then sell the milk to consumers (O'Ferrall-Berndt, 2003; MPO, 2016).

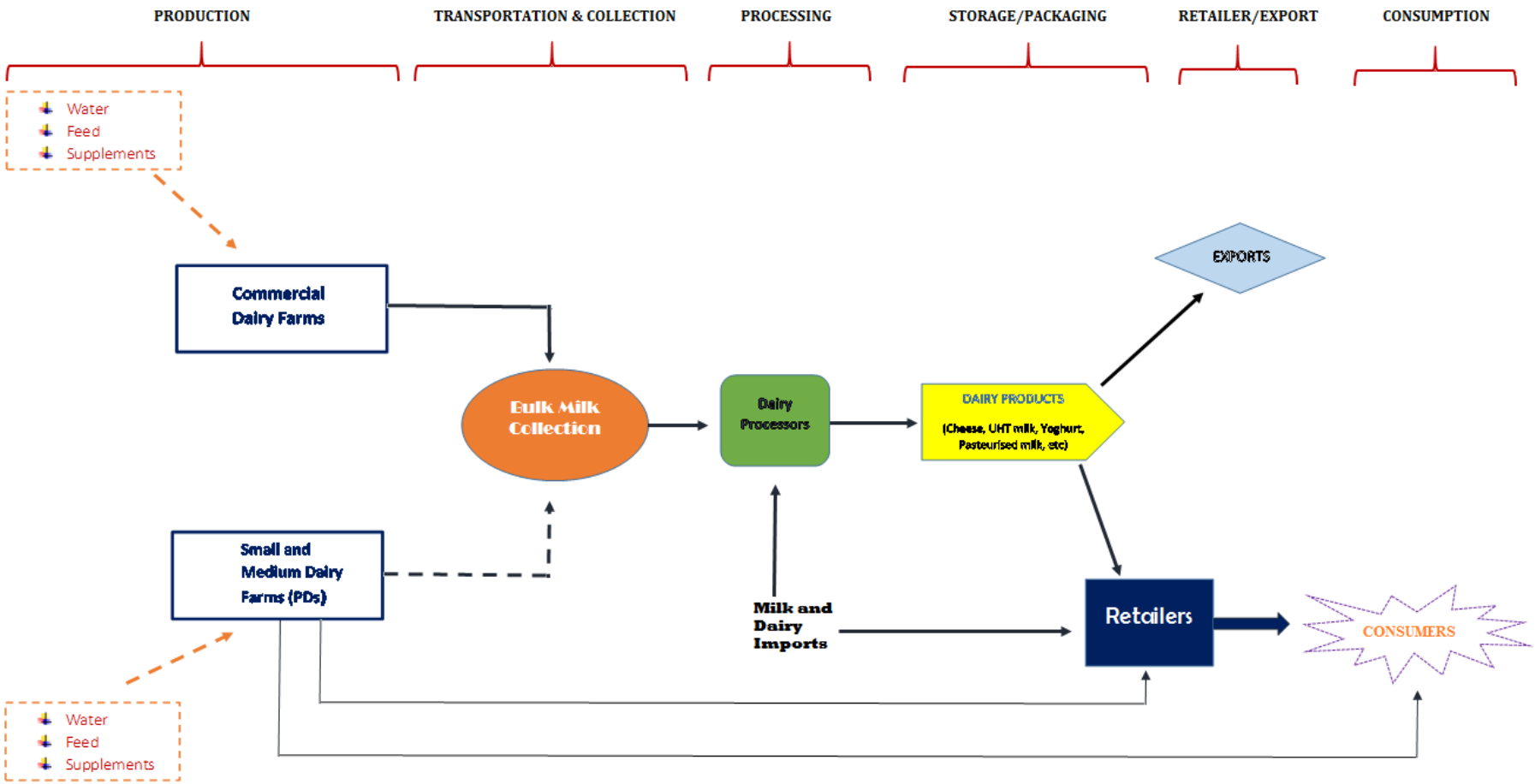

Figure 2.4: Structure of the milk and dairy value chain in South Africa.

*PDs – Producer-distributors.

The amount paid to the dairy farmer by the national distributors does not only depend on the volume received, but also on bacterial and somatic cell counts, as well as compositional quality (O'Ferrall-Berndt, 2003). A survey in 2003 by O'Ferrall-Berndt noted that milk received by national distributors often meet statutory requirements.

2.3.1.2. Milk from the informal value chain

Smaller unauthorised or unregistered milk producers constitute the informal milk value chain. Agenbag (2008) reports that most of the milk, raw and pasteurised, produced and sold in the informal sector does not meet the minimum statutory requirements, and the milking practices applied by this sector do not comply with best practice compliance standards. These producers also supply their milk directly to the communities through bulk milk tanks (Jansen, 2003; Agenbag, 2008). Safety of milk produced in the informal value chain was reported as a risk to public health, and this is evident from various studies conducted in SA between 2003-2012 (Jansen, 2003; O'Ferrall-Berndt, 2003; Cawe, 2006; Lues et al., 2003; 2010; 2012). This milk generally does not satisfy the microbiological and antibiotic residues statutory requirements (SA, 2001 Act (54), (1972)) and there are no penalties for poor quality. The only Act in SA which governs the safety of milk and sets the standards to which milk and dairy products must conform, is the Foodstuffs, Cosmetics and Disinfectants Act, No. 54 of 1972: Regulations relating to milk and dairy products. However, this Act does not contain a specific guideline that regulates the production, processing and supply milk in the informal sector. The Act is administered by the Directorate of Food Control of the Department of Health (O'Ferrall-Berndt, 2003). Table 2.2 presents the set microbiological standards for milk produced and sold to the public (SA, 2001 Act (54), (1972)).

2.3.2. Producer-distributor bulk milk (PDBM)

The direct sale of milk from the producer to the public is a common practice across the globe. This milk is sold either as raw or pasteurised PDBM. There is a growing number of unpasteurised milk consumers around the world who buy milk which is sold directly from producers on farms, retail shops or vending machines (Oliver et al., 2009; EFSA, 2015). Increased interest in raw milk consumption has been advocated for due to enhanced nutritional quality, taste, and health benefits, although no scientific-based data has substantiated these claims (Oliver et al., 2009). In the USA, 29 states out of about 50 allow the sale of raw PDBM (Jayarao and Henning, 2001; Jayarao et al., 2006; Oliver et al., 2009). In the European Union, the direct sale of milk is regulated by the national law of the member countries (Pyz-Łukasik et al., 2015). Epidemiological studies have shown clearly that raw milk can be contaminated by a variety of pathogens, some of which are implicated in disease outbreaks. Therefore, only state certified raw milk producers are allowed to sell raw milk directly to the public (Oliver et al., 2009; Pyz-Łukasik et al., 2015). PDBM in SA is vended raw or pasteurised. Both registered (formal) and unregistered (informal) milk producers fall under this type of milk value chain. People from the lower socio-economic level in urban and rural areas in SA buy the milk directly from producers. These points of sale have been defined as PD outlets in this study.

Only certified PDs are permitted by law in SA to sell raw milk directly to the public. However, research surveys have revealed that raw PDBM is sold by many unregistered and unauthorised PDs (Agenbag, 2008). Table 2.3 depicts a summary of PDBM quality in SA from 2003 to 2016 as reported by different studies from the same region or different geographical regions.

Table 2.2: South African national standards on milk quality (Foodstuffs, Cosmetics and Disinfectants Act (54), 1972).

Analysis	Raw milk before further processing	Raw milk directly to the public	Pasteurised milk
Total plate count cfu/ml	<200 000	<50 000	<50 000
Coliform count cfu/ml	<20	<20	<10
E. coli count cfu/ml	ND	ND	ND
Pathogens	ND	ND	ND

*ND – Not detected.

A survey conducted in 2003 on PDBM from a marginal urban settlement in central SA revealed that more than 60% of the milk samples exceeded the national standards in terms of total plate count, total coliform and *E. coli* counts (Lues et al., 2003). In the same year O'Ferrall-Berndt (2003) reported that, out of the 135 raw and pasteurised PDBM samples purchased from milk-shops (PD outlets) in urban and pre-urban areas of Pretoria in SA, 87% of the samples were not fit for human consumption on the basis of the minimum standards prescribed in the Foodstuffs, Cosmetics and Disinfectants Act (Act 54 of 1972) (Table 2.2). O'Ferrall-Berndt (2003) also detected *Staphylococcus aureus* and *S. aureus* enterotoxins in 40.0% and 7.8% of the milk samples, respectively (Table 2.3). Despite high *E. coli* counts in some cases, the PDBM sold at different selling points around the Mangaung Area (SA) in 2006 did not contain any of the tested pathogens (*S. aureus, Bacillus cereus* and *Clostridium botulinum*) (Cawe, 2006). In 2014 and 2016, studies conducted on bulk milk at dairy farms in the Eastern Cape Province of SA revealed the presence of *E. coli* , belonging to enteropathogenic *E. coli* (EPEC), EHEC, enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely

adherent *E. coli* (DAEC) and uropathogenic *E. coli* (UPEC) (Caine et al., 2014; Msolo, 2016) (Table 2.3). The study by Msolo (2016) also reported the presence of antibiotic resistant *E. coli* O157:H7 in raw PDBM. All these studies in SA (Table 2.3), highlighted sanitation and hygiene of the farm environment, milking and storage equipment, as well as personnel hygiene as the main cause of microbial inadequacies in the quality of PDBM sold directly to the public. The studies also showed that dairy farms are reservoirs of pathogenic *E. coli* serotypes, which are a cause for concern in public and environmental health.

2.3.2.1. Production, processing and sale of PDBM

In different parts of the world, PDBM is produced and sold at farms, retail shops, vending machines or via the internet (EFSA, 2015; Kamana, 2015). In SA, PDBM is produced at small or medium dairy farms (MPO, 2016). PDBM in SA is typically (i) raw milk for human consumption, (ii) raw milk received with the intention of processing and selling as pasteurised milk, and (iii) milk that has been pasteurised elsewhere at an approved facility. PD outlets usually market their products as being "fresh milk" or "fresh farm milk", giving the impression that it is safe (Agenbag, 2008). Consumers either bring containers to be filled directly from the bulk tank or buy small plastic containers (1 to 5 L) prefilled with bulk milk at the PD outlets. The premises where PD outlets are located often range from small depots in shopping centers to fruit and vegetable shops, supermarkets and general dealers (Jansen, 2003; O'Ferrall-Berndt, 2003; Agenbag, 2008). One safety challenge about the sale of PDBM is that many PD outlets owners do not have sufficient technical and scientific knowledge in both dairy science and dairy microbiology, for large scale collection and distribution of saleable milk (Agenbag, 2008).

Table 2.3: Summary of producer-distributor bulk milk quality in South Africa, from 2003 to 2016.

Year	Milk type	Microbial indicators				Possible pathogenic organisms	References
		TPC	**Coliform**	***E. coli***	**Enterobacteriaceae**		
2003	Raw PDBM	(96.7%) 10^6 – 10^{10} cfu/ml	(100%) 10^2 – 10^9 cfu/ml	(23.3%) 10^4 – 10^6 cfu/ml	(100%) 10^5 – 10^9 cfu/ml	Not tested	(Lues et al., 2003)
2003	[a]PDBM	(95.6%) 1.0 x 10^2 – 2.7 x 10^7 cfu/ml	(32.0%) 20 – 3.4 x 10^5	17.7%	Not tested	*Staphylococcus aureus* (40.0%) *S. aureus* enterotoxins (7.8%)	(O'Ferrall-Berndt, 2003)
2006	[a]PDBM	(100%) 10^3 – 10^8 cfu/ml	(63.0%) 10^5 – 10^7 cfu/ml	(23.8%)10^4 – 10^6 cfu/ml	Not tested	Not detected	(Cawe, 2006)
2010	Raw PDBM	(93.9%) 10^4 – 10^7 cfu/ml	(100%) 10^2 – 10^4 cfu/ml	(87.8%) 10 – 8.3 x 10^3 cfu/ml	Not tested	Not tested	(Lues et al., 2010)
2014	Raw PDBM	Not tested	Not tested	44 -50%	Not tested	EHEC, EIEC, EAEC, EPEC, ETEC, UPEC	(Caine et al., 2014)
2016	Raw PDBM	Not tested	Not tested	Not tested	Not tested	*E. coli* O157:H7	(Msolo, 2016)

*Percentages represent proportions of PDBM samples which were above South African national standards (SA, 2001 Act (54), (1972)) on milk quality sold to the public and the concentration range.
*[a]PDBM represents both raw and pasteurised.
*TPC – Total plate count.

2.3.2.2. Consumption patterns of PDBM

Data on consumption of PDBM is very scarce. As a result of the economic depression in SA, the attitude of consumers is governed by their financial status (Altman et al., 2009). PDBM is produced in all the provinces of SA and is affordable to the poor and vulnerable, although it is often of compromised quality (O'Ferrall-Berndt, 2003; Agenbag, 2008). Milk from national distributors is of high quality and the high prices of commercially pasteurised milk forces many consumers to buy PDBM (O'Ferrall-Berndt, 2003). There are no official statistics of PDBM consumption patterns in SA, although more than 90% of households in urban areas consumes milk on a daily basis as an important basic foodstuff (Nel and Steyn, 2002). Due to lack of refrigeration facilities for the rural population of SA, the consumption patterns of milk is not clear.

2.3.2.3. PDBM contamination routes

Several factors of PDBM contamination have been reviewed, which include animal and equipment cleanliness, feeding and housing strategies, rinsing water for milking machines and equipment, mastitis in animals, transportation and storage conditions (Reinemann et al., 2003; Torkar and Teger, 2008). The potential sources of on-farm contamination and their relationship to each other are shown in Figure 2.5. Microorganisms present in raw milk may include normal flora and pathogenic microorganisms. Freshly drawn milk from the udder of a healthy cow possesses temporary germicidal or bacteriostatic properties, which halt the growth of microorganisms (Murphy and Boor, 2000). Therefore, the small number of microorganisms present in the milk are of little importance commercially and present no danger to the consumer (Cawe, 2006).

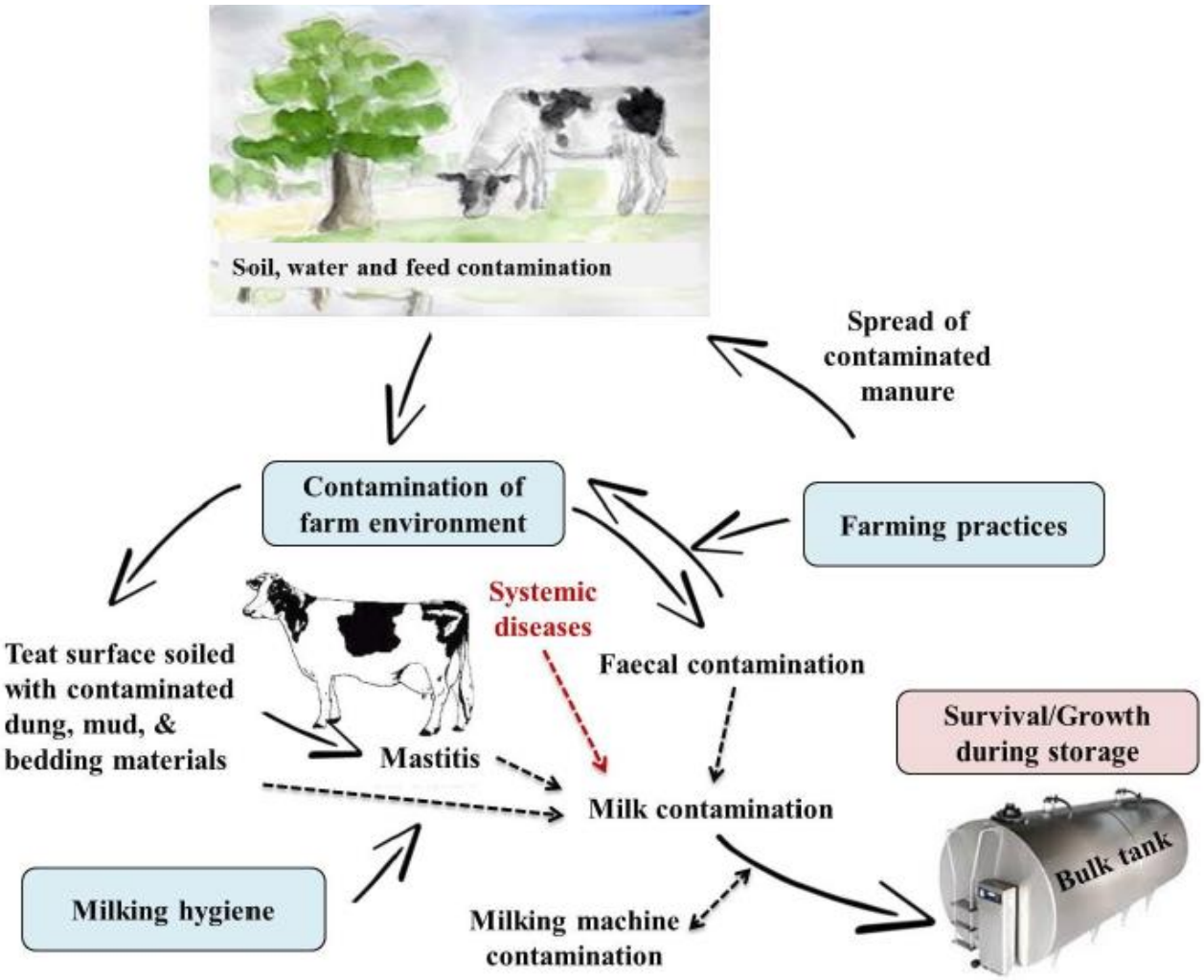

Figure 2.5: Schematic diagram for the potential sources of PDBM contamination on dairy farms (EFSA, 2015).

Dirt, such as cattle faeces, soil and other materials surrounding the dairy farm environment, have been discussed as the main source of milk contamination. The dirt attaches to the exterior of the udder and can also enter the teats and cause mastitis (Frank, 2007). Studies have shown that increased milkings from the first lactation places stress on the teats and mammary glands by the very large amounts of milk produced and the milking machine can cause teat canals to become more open (Figure 2.6). The stress may open the teat canal for the entry of bacteria capable of infecting the glands and cause mastitis (Ledenbach and Marshall, 2010).

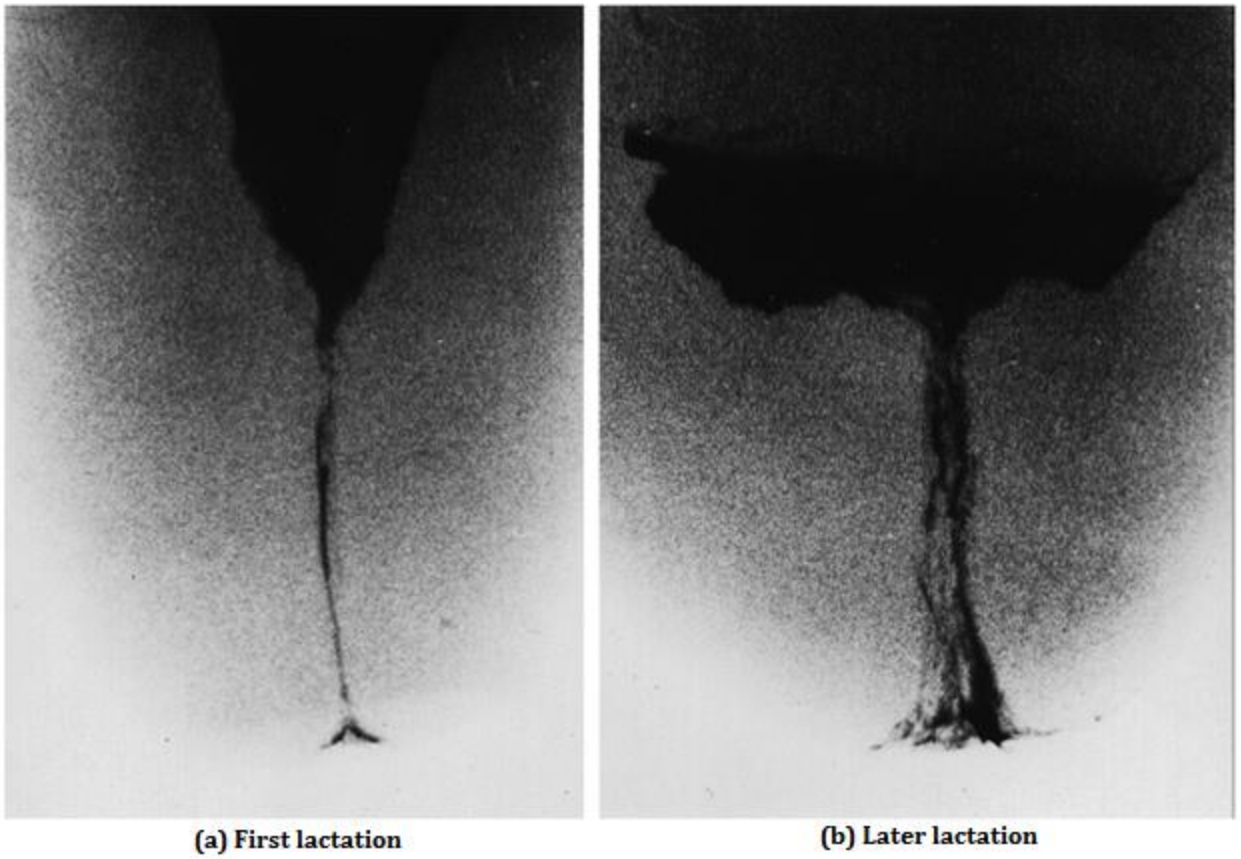

Figure 2.6: X-ray photographs showing an increase in the diameter of the teat canal of the same teat of a milking cow between (a) the first lactation and (b) a later lactation (Ledenbach and Marshall, 2010).

A review by Godkin and Leslie (1993) revealed that bacterial populations in bulk tank milk consist of a variety of bacterial species and these reflect their origin or source. *S. aureus* and *Streptococcus agalactiae*, if identified on a culture of a sample of bulk milk, can be reliably traced back to cows suffering from mastitis (Godkin and Leslie, 1993). Presence of pathogenic STEC in PDBM is generally as a result of two suggested routes (1) rare sub-clinical mastitis causing STEC excretion from the udder and (2) faecal routes (directly or indirectly) (Farrokh et al., 2013). Farrokh et al. (2013) also report that lactating dairy animals carry potentially pathogenic STEC in their

intestines, which is excreted in their faeces and in turn soils the teats, subsequently becoming a source of contamination during the milking process.

Cleanliness of milking equipment reduces microbial contamination in the dairy industry. Insufficiently cleaned milking equipment can form fouling films (multi-species biofilms), which adhere to surfaces of equipment and may become reservoirs of milk contaminants (Kumar and Anand, 1998; Chmielewski and Frank, 2003). Given that milking machines exert a vacuum on the teats during milking and air often leaks into the system, bacteria on the surfaces of the cow or in water retained from pre-milking preparation can be drawn into the milk (Ledenbach and Marshall, 2010). Although this is insignificant under normal production conditions the concentration of microorganisms can further increase due to their growth.

PDBM can be contaminated after processing. Bacteria recontaminating pasteurised milk originate primarily from water aerosols and air in the filling equipment or improperly cleaned processing and handling equipment (Eneroth et al., 2000; Ledenbach and Marshall, 2010). A recent study has also shown that certain species of bacteria harbour heat resistant genetic determinants, which can also be a cause of contamination in pasteurised PDBM (Mercer et al., 2015). Also, exposure of pasteurised milk to environmental air during filling can give rise to post-pasteurisation recontamination (Ledenbach and Marshall, 2010). Contamination of pasteurised milk by raw milk is also a source of bacterial recontamination in processed milk. Spores of bacteria can survive ultra-heat treatment and pasteurisation of milk. The spore forming bacteria (genera *Bacillus*) are abundant in dust, dairy feed concentrates and forages. Therefore, they are often present on the skin and fur of cattle from which they can enter milk (Ledenbach and Marshall, 2010). Given suitable conditions, the spores have the ability to germinate and cause spoilage.

2.4. FOOD SAFETY IN DAIRY

The dairy industry today has established efficient production processes and a wide product distribution network, and in this regard, there is need to enhance consumer safety against milk-borne pathogens (Yazdankhah et al., 2004). High production of, and trade in, milk and its products have also provided a route for the rapid and efficient dispersal of microorganisms to a large population of consumers, if any products are contaminated (Yazdankhah et al., 2004; Asselt et al., 2017). This can lead to the introduction and establishment of new diseases in geographical areas that have never experienced the foodborne pathogens (Yazdankhah et al., 2004; Oliver et al., 2005; Farrokh et al., 2013; Asselt et al., 2017). Preventing illnesses and deaths associated with milk-borne pathogens remains a major public health challenge, globally (Oliver et al., 2005). Milk is rich in a variety of nutrients, has neutral pH and high water activity, and these promote growth of many spoilage and pathogenic microorganisms (Ledenbach and Marshall, 2010).

Milkborne diseases have occurred ever since cows were milked and in the past, the commonly reported diseases from milk were tuberculosis, brucellosis, and scarlet fever (Yazdankhah et al., 2004). Today, there are several existing and emerging milk-borne diseases caused by several pathogenic microorganisms, which are of public health significance. Newell et al. (2010) report that these milk-borne diseases are expected to decline owing to improvements in dairy technology as well as food safety management systems in dairy production. However, several milk-borne disease outbreaks are still experienced across the globe to-date, mainly because the microbiological safety of dairy remains dynamic and is heavily influenced by multiple factors along the food chain from ‘farm to fork’ (Newell et al., 2010). The predominant human bacterial pathogens of concern in dairy, including *Salmonella* spp., *L. monocytogenes*, *S. aureus*, *Campylobacter* spp. and pathogenic *E. coli*, can be transferred from milk and other dairy products

to humans (Newell et al., 2010; Farrokh et al., 2013; EFSA, 2015; Asselt et al., 2017). The knowledge that these bacterial pathogens are disseminated through milk and other foods comes from the fact that they have well recognisable clinical symptoms in cases of disease outbreaks. Other pathogenic microorganisms are still of public health significance in dairy, although little is known about their spread and colonisation because they do not cause easily recognised conditions with distinct microbiological diagnoses (Newell et al., 2010).

Table 2.4 presents the prevalence of milk-borne pathogens isolated from milk, which can be transmitted from milk to humans. Numerous surveys around the globe have detected foodborne pathogens in bulk tank milk which are of public health concern (Table 2.4). Prevalence of these foodborne pathogens in milk, including *C. jejuni*, STEC, *L. monocytogenes*, and *Salmonella* spp. varies significantly. The most prevalent human pathogens found in raw and pasteurised milk include *S. aureus*, STEC (O157 and non-O157), *Campylobacter* spp., *L. monocytogenes* and *Salmonella* spp. (Table 2.4).

Oliver et al. (2005), who reviewed the factors that influence the contamination and the prevalence of foodborne pathogens in milk, reported the following: (i) farm size, (ii) number of animals on the farm, (iii) hygiene, (iv) farm management practices, (v) variation in sampling and types of samples evaluated, (vi) differences in detection methodologies used, (vii) geographical location, and (viii) season.

From 2006 to 2016, several outbreaks of diseases, including HUS, campylobacteriosis, salmonellosis and listeriosis, that were related to the consumption of unpasteurised and pasteurised milk or dairy products have been reported across the globe (Table 2.5). However, few epidemiological statistics have implicated food contamination in disease outbreaks in Africa, even

though there is a globalised network of food (including dairy) trade. Table 2.5 shows that, from 2006 to 2016, campylobacteriosis and STEC infections/HUS accounted for more outbreaks and number of cases than other diseases. A review by Oliver et al. (2009) also reports similar milk-borne outbreaks from 2000 to 2008 in the USA. Most reports on milk-borne disease outbreaks in the world have implicated unpasteurised milk, although some epidemiological studies have also pronounced pasteurised milk due to inadequate pasteurisation processing or post-pasteurisation contamination (Table 2.5). Different food safety authorities around the world have banned the sale of unpasteurised milk and raw milk products as they pose a great risk to consumers (D'amico et al., 2008). However, there are raw milk advocates who believe that there are more benefits, than the taste, in consumption of raw than heat treated milk. An estimated 25% of the food and water borne diseases has been linked to milk and its products (Oliver et al., 2009).

2.4.1. Important milk-borne microbial contaminants

The control of spoilage and pathogenic microflora along the milk value chain is an important prerequisite for sustainable trade and public health.

2.4.1.1. Spoilage microbiota

The vast majority of bacteria that contaminate milk are nonpathogenic, although they are of particular concern to the dairy industry because of their effect on product quality and subsequent economic impacts (Cawe, 2006). Nonpathogenic or spoilage microorganisms of concern in dairy include aerobic psychrotrophic Gram-negative bacteria, yeasts, molds, heterofermentative lactobacilli and spore-forming bacteria. Psychrotrophic microorganisms are of particular importance in the spoilage of milk and dairy products (Ledenbach and Marshall, 2010). *Pseudomonas* spp. are the major psychrotrophic microorganisms implicated in the degradation of

milk components through various enzymatic activities and the subsequent reduction in the shelf-life of processed milk (Ledenbach and Marshall, 2010). Spore-forming bacteria are divided into two main genera, which are *Bacillus* (comprises aerobic and facultative anaerobic species) and *Clostridium* (contains mainly obligate anaerobic species) (Cawe, 2006). Erkmen and Bozoglu (2016) report that fungal spoilage of dairy foods is manifested by the presence of a wide variety of metabolic by-products, causing off-odours and flavours, in addition to visible changes in colour or texture. Gassing defects in cheeses is caused by coliforms, yeasts, heterofermentative lactic acid bacteria and spore-forming bacteria (Ledenbach and Marshall, 2010).

Table 2.4: Prevalence rates of milk-borne pathogens (2006 – 2016) isolated from bulk milk.

Organism	Disease	Symptoms	Prevalence (%) rates in bulk milk	Reference
Listeria monocytogenes	Listeriosis	Fever, muscle aches, and sometimes nausea or diarrhea	1.6 – 10.0	(Jayarao et al., 2006; Hamdi et al., 2007; D'amico et al., 2008; Bardon et al., 2012; Bianchi et al., 2013)
Salmonella spp.	Salmonellosis	Nausea, vomiting, abdominal cramps, diarrhea, fever, chills, headache, blood in the stool	0.3 – 48.0	(Jayarao et al., 2006; Houser et al., 2008; Van Elsas et al., 2011; Bardon et al., 2012; Mallet et al., 2012; Bianchi et al., 2013)
Campylobacter jejuni	Campylobacteriosis	Cramping, abdominal pain, fever, nausea and vomiting, watery diarrhea, sometimes bloody	0.5 – 12.0	(Jayarao et al., 2006; Hussain et al., 2007; Messelhaeusser et al., 2008; Bardon et al., 2012; Bianchi et al., 2013; Bianchini et al., 2014)
Escherichia coli O157:H7 & non-O157 STEC	Haemorrhagic colitis, haemolytic uraemic syndrome (HUS)	Diarrhoea, kidney failure	0.2 – 15.2	(Jayarao et al., 2006; Karns et al., 2007; Murphy et al., 2007; Cobbold et al., 2008; Solomakos et al., 2009; Van Kessel et al., 2011; Bardon et al., 2012; Bianchi et al., 2013; Caine et al., 2014; Msolo, 2016)
Yersinia enterocolitica	Yersiniosis	Diarrhea, abdominal pain, vomiting	1.2 – 47.3	(Jayarao et al., 2006; Yucel and Ulusoy, 2006; Hanifian and Khani, 2012)
Staphylococcus aureus	Staphylococcal enterotoxin poisoning	Nausea, violent vomiting, abdominal cramping, with or without diarrhea	56.6 – 100.0	(Gündoğan et al., 2006; Peles et al., 2007; Riekerink et al., 2010)

Mycobacterium avium subsp. *paratuberculosis*	Crohn's disease	Diarrhea, abdominal pain and cramping, blood in stool	88.6	(Ricchi et al., 2016)
Mycobacterium bovis	Tuberculosis	Coughing that lasts three or more weeks, coughing up blood, chest pain, or pain with breathing or coughing, unintentional weight loss, fatigue, fever, night sweats, chills.	1.9	(Riekerink et al., 2006)
Brucella spp.	Brucellosis	Fever, chills, loss of appetite, sweats, weakness, fatigue, joint, muscle and back pain, headache.	18.3	(Aulakh et al., 2008)
Streptococcus spp.	Streptococcal infections	Throat pain, red and swollen tonsils, fever	1.0 – 29.0	(Østerås et al., 2006; Tenhagen et al., 2006)

Table 2.5: Summary of milkborne diseases recorded in different parts of the world from 2006 to 2016.

Organisms	Year	Country	Milk type	Details of the outbreak	Reference
Listeria monocytogenes	2007	Germany	Pasteurised milk	189 patients hospitalised	(Koch et al., 2010)
	2009	USA	Pasteurised milk	8 cases, 7 of these were pregnant women and one was a 3-year-old	(Jackson et al., 2011)
	2008	USA	Pasteurised milk	5 cases	(CDC, 2008)
Pathogen *E. coli* (STEC O157 and non-O157)	2008	Germany	Raw milk	1 outbreak, 23 cases	(EFSA, 2015)
	2012	Finland	Raw milk	1 outbreak, 8 cases	(EFSA, 2015)
	2008	USA	Raw milk	2 cases (children)	(Guh et al., 2010)
	2006	USA	Raw milk	85 cases, 36 hospitalised	(CDC, 2008b)
	2006	USA	Raw milk	6 cases	(Denny et al., 2008)
	2013	Italy	*Milk	20 cases, children median age 17	(Germinario et al., 2016)
	2016	Italy	*Milk	25 cases, 19 developed HUS, 3 died	(ECDC and EFSA, 2016)
Campylobacter jejuni	2007	USA	Raw milk	68 cases, 2 hospitalised	(KDHE, 2007a)
	2007	Denmark	Raw milk	2 outbreaks, 12 cases	(EFSA, 2015)
	2007	The Netherlands	Raw milk	1 outbreak, 18 cases	(EFSA, 2015)
	2007	Finland	Raw milk	1 outbreak, 4 cases,	(EFSA, 2015)
	2007	Germany	Raw milk	1 outbreak, 14 cases	(EFSA, 2015)
	2008	USA	Raw milk	16 cases, 2 hospitalised	(CDPH, 2008)
	2011	Germany	Raw milk	3 outbreaks, 32 cases	(EFSA, 2015)
	2012	Finland	Raw milk	2 outbreaks, 22 cases	(EFSA, 2015)
	2017	Sweden	Raw milk	11 cases, seven of them were 2 to 7-year old children	(Lahti et al., 2017)
Salmonella spp.	2006	USA	Raw milk	85 cases, 36 hospitalised	(CDC, 2008b)
	2007	USA	Raw milk	25 cases	(KDHE, 2007)

*Milk – Either pasteurised, unpasteurised milk or milk products.

2.4.1.2. Pathogenic microbiota

A lot of pathogens (bacteria, virus, fungi and parasites), known to cause fatal diseases in humans, are transmitted through contaminated food. Contaminated milk is among the food implicated in disease outbreaks across the globe. Pathogenic microorganisms contaminate milk as a result of poor milk handling along the dairy value chain. More than 90% of all reported cases of milk and dairy related illnesses are associated with bacterial agents (Oliver et al., 2009). These bacterial agents belong to different families, genera and species. Some of the most common bacterial agents of concern in milkborne outbreaks, which are also depicted in Tables 2.4 and 2.5, belong to Enterobacteriaceae, Listeriaceae, Campylobacteraceae and Staphylococcaceae families. Bacteria belonging to the Enterobacteriaceae family are mostly implicated as spoilage microbiota in dairy, although some have evolved into opportunistic pathogens as a result of genetic transfers within food sources such as milk (Gilchrist et al., 1995). Of late, pathogenic *E. coli* has become a global foodborne pathogen, causing serious fatalities in both developed and developing countries (Oliver et al., 2009). Several foods have been implicated as vehicles of pathogenic (diarrheagenic) *E. coli* which include processed and unprocessed vegetables (Franz and van Bruggen, 2008), ground beef and hamburgers made from beef (Bell et al., 1994; Duffy et al., 2006) and sea food (Samadpour et al., 1994).

2.5. *ESCHERICHIA COLI*

E. coli belongs to the Enterobacteriaceae family within the class of Gamma Proteobacteria (Kersters et al., 2006). It is a Gram-negative, non-spore-forming, facultative anaerobic bacterium of the genus *Escherichia.* *E. coli* can grow at a temperature range of 7 to 46 °C with an optimum of 37 °C. *E. coli* is an important part of the healthy gut and so can be described as a component

of host innate defenses. It is a commensal and harmless bacterium that co-exists in a mutually beneficial relationship with its animal host, including man, in the intestinal microflora. *E. coli* provide vitamin K_2 for its host, which is required for posttranslational modification of proteins involved in blood coagulation and bone metabolism and act as a protective agent against pathogenic bacteria (Sharma et al., 1992). Despite the mutual benefit, few strains of *E. coli* can cause debilitating and fatal diseases, especially in immunocompromised human hosts (Buchanan, 1997; Constantiniu, 2002; CDC, 2007; Control and Prevention, 2007). Different strains of *E. coli* present different pathogenic determinants, which may determine the pathogenic functions of the bacterium and clinical syndrome with distinctive pathological and epidemiological characteristics of disease (Robins-Browne, 2004).

Traditionally, *E. coli* has been identified based on its biochemical activities. To-date a combination of biochemical, proteomics and molecular techniques have been used to identify the organism (Mazzeo et al., 2006; Farzan et al., 2012). *E. coli* is still screened biochemically, by the use of selective media or indicator media which use specific fermentative growth characteristics and resistance to antimicrobial compounds. Bacterial species have since been predicted phenotypically by the use of kits. New technologies, which use proteomics, such as Matrix-associated laser desorption and ionisation time of Flight (MALDI-TOF) have been adopted for rapid typing of *E. coli* and other bacterial species, based on their phenotypic characteristics (Mazzeo et al., 2006). Rapid advances in mass spectrometry, bioinformatics and protein separation technologies have produced a step change in current proteomic capabilities to improve phenotypic typing schemes (Maja et al., 2013).

Distinct phenotypic variations within a species of bacteria can further subdivide or classify bacteria into serotypes. Serotyping in *E. coli* uses surface antigens O (part of lipopolysaccharide layer), H

(Flagellin) and K (capsule) (Kauffmann, 1947). However, a specific combination of O and H antigens defines the serotype of an *E. coli* isolate (Kaper and O'Brien, 1998). More than 400 O and H antigents are recognised currently (Machado et al., 2000). Determination of serotypes can be done using agglutination antisera or by molecular methods using conventional or novel PCR techniques. Genomic DNA of *E. coli* is extracted and the *fliC* gene is used for H typing, while the *rfb* gene is used for O typing (Coimbra et al., 2000; Machado et al., 2000). The O-antigen maybe lost under laboratory conditions and this leaves most *E. coli* isolates untypeable by serological methods (Coimbra et al., 2000). Serotyping is an expensive process and is only performed in a few reference laboratories. Therefore, cheaper alternatives such as the use of molecular techniques have been adopted by different researchers during *E. coli* serotyping (Coimbra et al., 2000; Machado et al., 2000).

Genotypic characterisation of *E. coli* has great importance clinically and in epidemiological studies of outbreaks. Advances in bacterial genomics and genomic analysis are making it possible for DNA-based typing procedures to be developed for the classification of bacterial isolates. Improvements in the polymerase chain reaction (PCR) techniques and DNA sequencing/ microarray technology have proven to be more useful in classifying *E. coli* for epidemiological and phylogenetic analyses than phenotypic methods (Persson et al., 2007). Conventionally, 16S ribosomal RNA-encoding gene sequences and pulsed-field gel electrophoresis (PFGE) were used as standard methods for typing bacteria. Multi-locus sequence typing (MLST) and multi locus variable number tandem repeat analysis (MLVA) have been developed for *E. coli*, based on the sequence types of 7 housekeeping genes (Wirth et al., 2006). Analysis of band patterns generated by randomly amplified polymorphic DNA (RAPD), coupled with PFGE has been used to determine genetic diversity among *E. coli* isolates. PCR can be coupled with PFGE, RFLP and

RAPD in genetic typing (relatedness, virulence factors, O and H serotypes) of *E. coli* isolates (Coimbra et al., 2000).

2.5.1. Pathogenic (diarrheagenic) *E. coli*

The recognised pathogenic *E. coli* strains include certain types which may establish enteric infections and those which may establish extraintestinal infections (Kaper et al., 2004). This review focuses on the diarrheagenic *E. coli* associated with food and which can cause enteric infections. The virulence mechanisms of each *E. coli* pathotype are determined by the carriage of key virulence determinants (Kaper et al., 2004). The virulence determinants promote colonisation of the host, cellular adherence/invasion, evasion of host defenses and disruption of host cell signaling pathways (Kaper et al., 2004). Pathogenic *E. coli* implicated in foodborne (including milk and dairy) diarrheal diseases are classified into six major groups, on the basis of serological characteristics and virulence properties: EPEC, EHEC, ETEC, EAEC, EIEC and diffusely adherent *E. coli* (DAEC) (Kaper et al., 2004). Table 2.6 depicts the serotype and disease characteristics, as well as the virulence factors and mechanisms of diarrheagenic *E. coli* categories implicated in foodborne outbreaks. Each class further falls within a serological subgroup and manifests distinct features in pathogenesis.

Problems associated with *E. coli* and its pathotypes in milk and dairy remain prominent even in countries with sophisticated dairy technology, food safety and quality systems in place (CDC, 2005; EFSA, 2015). A review by Okeke (2009) reports that diarrheagenic *E. coli* have been associated with diarrheal diseases in different parts of Africa, particularly among young children, HIV-positive individuals and visitors from abroad. Outbreaks caused by pathogenic *E. coli*, in particular, STEC, which is associated with milk and central to this research, have been well

documented and reported across the globe (CDC, 2005; EFSA-ECDC, 2012; EFSA, 2015). Table 2.5 documents some of the milkborne outbreaks associated with STEC from 2006 to 2016. STEC has been reported and implicated in more foodborne disease outbreaks in comparison to other diarrheagenic *E. coli.* Furthermore, STEC has the potential to cause the most severe clinical symptoms (Kaper et al., 2004). It is believed to be the second most frequent implicated pathogens after rotavirus and the main cause of diarrheal illness among bacterial pathogens. The elimination of *E. coli* in contaminated milk by means of pasteurisation is the internationally recognised and adopted measure to ensure public health and protection from milk-borne STEC infections. However, most recent studies have shown that the organism can resist pasteurisation temperatures depending on the food matrix (Mercer et al., 2015). Establishing the environmental niche or source of *E. coli* for milk has always been a challenge for many researchers. Nonetheless, dairy cattle appear to be the major reservoir for this pathogenic *E. coli*, which asymptomatically carry the bacterium (Farrokh et al., 2013).

2.5.2. STEC

STEC, which belongs to the EHEC group, was first recognised as a cause of human disease in 1982 although reports of hemorrhagic colitis (bloody diarrhoea) and haemolytic uremic syndrome (non-bloody diarrhoea) associated with *E. coli* first appeared in the literature in 1983 (Okeke, 2009). O'Brien et al. (1983) report that *E. coli* O157:H7 isolates from cases of HUS produced a toxin, which was later shown to be phageborne and identical to the *Shigella dysenteriae* type I shigatoxin (Stx). The toxin produced by the *E. coli* has cytotoxic effect on Vero cells, hence the organisms were termed verocytotoxin or shigatoxin producing *E. coli*, owing to the fact that it produces a toxin identical to *Sh. dysenteriae*. In the same year, Riley et al. (1983) isolated a rare *E. coli* O157:H7 serotype from two outbreaks in the USA. The illness was characterised by severe

crampy abdominal pains, initial watery diarrhea, followed by gross bloody diarrhea, and little or no fever, which are typical of haemolytic colitis (HC) (Nataro and Kaper, 1998). Karmali (1989) later reported sporadic cases of HUS with faecal cytotoxin and cytotoxin producing *E. coli* in stools. HUS later develops into acute renal failure (thrombocytopenia, and microangiopathic haemolytic anaemia) (Nataro and Kaper, 1998). Since then, more than 400 different O and H serotypes have been identified, although a smaller proportion has been linked to human illnesses (Okeke, 2009). EHEC pathogens are zoonotic and have since been associated with pandemic food and water borne illnesses across the globe, with serotype O157:H7 being implicated in the majority of the illnesses, in contrast to the non-O157 counterparts (Constantiniu, 2002).

2.5.2.1. Detection of STEC in food

Very few studies have quantified STEC in food. The presence of STEC has since been detected qualitatively in food samples. In addition to being diverse and differing greatly in both physiological characteristics and pathogenic potential to humans, STECs also have different detection methods. Since STEC O157 has been well documented and is the most prevalent cause of human disease and outbreaks, its detection methods are well developed as well and widely available (Farrokh et al., 2013). In view of this, other emerging EHEC subtypes (non-O157) are unaccounted for or are under reported due to lack of available or well developed detection methods. Nevertheless, emerging non-O157 STEC serotypes of *E. coli* have become recognised in causing deadly HC and HUS associated with food and water in different geographical regions of the world. STEC cells are known to enter a dormant state in food; they are still viable but non-culturable (Dinu and Bach, 2011). Therefore, direct culturing of a food sample might give a false negative result, although the cells may still be viable and pathogenic. Farrokh et al. (2013) and Nataro and Kaper (1998) reviewed the methods of detecting STEC using phenotypic typing assays

(biochemical activities) and documented the following: selective agars, immunomagnetic separation and latex agglutination assays, colony hybridisation and immunoblot assays. Molecular typing methods have been employed in characterising STEC serotypes. Discussed PCR-based methods such as sequencing of the 16S RNA gene, MLST, RFLP, RAPD and PFGE based methods have been used in the characterisation of both O157 and non-O157 STEC (Wirth et al., 2006). In several studies, the isolation of STEC from milk used qualitative methods, which combine phenotypic and genotypic typing (Momtaz et al., 2012; Caine et al., 2014; Msolo, 2016). Trevisani et al. (2013) identified and estimated the number of STEC O157 and O26 using the most-probable-number-PCR method.

2.5.2.2. Sources and routes of STEC in milk

A large number of cases of human illnesses caused by STEC worldwide has raised safety concerns for foods of bovine origin, which are milk and beef. The main route by which STEC enters raw milk is though faecal contamination or intermammary secretion from cow udders (King, 2007). Farrokh et al. (2013), in their review article, documented that cattle provide a reservoir for STEC, which is important for its transmission to humans and STEC contamination of dairy products during processing in the dairy plant is rare. STEC can cause diarrhoea in very young calves but is non-pathogenic in adult cattle (Gyles, 2007; Clark, 2009). In a review study in Canada, Gyles (2007) reported that the prevalence of STEC in cattle faeces ranged from 0 to 71% and 0 to 100% of individual animals and animal herds, respectively. EFSA, in 2009, documented that the average proportion of STEC positive samples, based on the investigation of faeces from 5368 animals, was 2.2% and ranged from 0% to 30%. Recent studies in South Africa (Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2011; Iweriebor et al., 2015) have detected a wide range of estimates for the prevalence of healthy carriage of STEC in cattle, ranging from 27.7 to 31.7%. *E. coli* O157:H7

has been found to colonise at the terminal rectum of cattle in preference to other sites in the bovine gastrointestinal tract and this colonisation is the major feature of "supershedders" of STEC in faeces (Gyles, 2007). However, factors contributing to intermittent excretion of STEC by cattle include age, housing, stress, animal health, geographical area and previous contamination with STEC strains or other pathogens (Farrokh et al., 2013). Suffice to say, the bovine gastrointestinal tract provides a refuge for the propagation of STEC serotypes. Milk can be contaminated with cattle faeces (as a result of poor milk handling), which contain STEC and this poses a significant health risk to humans (Clark, 2009). Figure 2.8 depicts a cow being milked and there is faecal contamination of the udder, hind limbs and the milking equipment. The faeces have a potential of soiling the milk at this stage posing a risk of pathogenic contamination. This calls for control of STEC in animal reservoirs at the dairy farm to ensure public health safety. Albeit the contamination of milk by pathogens such as STEC is undoubtedly through faecal contamination, some published reports implicate intra-mammary source (pre/sub-clinical mastitis), although this is controversial (Gyles, 2007; Clark, 2009; Farrokh et al., 2013). However, STEC has been isolated from cattle with mastitis (Lira et al., 2004).

2.5.2.3. Epidemiology and clinical manifestation of STEC infection

STEC, in particular O157:H7, have been reported to cause illnesses even in healthy adults at a very low infectious dose (5 – 50 cells) (Delignette-Muller et al., 2008). The young, immunocompromised and elderly are at a particular risk of the progression of the disease to more severe sequelae yet in healthy adults, infection may be restricted to watery diarrhoea or may even be sub-clinical (Clark, 2009).

Table 2.6: Serotypes and disease characteristics, and virulence factor mechanisms of diarrheagenic *E. coli* categories (Clark, 2009).

Pathotype	**Common O-serotypes**	**Disease characteristics**	**Virulence factors and mechanisms**
Enteropathogenic *E. coli* (EPEC)	O26, O55, O86, O88, O103, O111, O119, O125ac, O126, O127, O128ab, O142, O145, O157, O158	Watery diarrhoea	Initial localised adherence via bundle-forming pili (BFP) followed by intimate adherence
Enterotoxigenic *E. coli* (ETEC)	O6, O7, O8, O9, O11, O15, O17, O20, O21, O25, O27, O29, O48, O55, O56, O63, O64, O65, O71, O73, O77, O78, O85, O86, O88, O105, O114, O115, O119, O126, O128ac, O133, O138, O139, O141, O147, O148, O149, O153, O159, O166, O167	Watery diarrhoea	Heat labile/heat stable enterotoxin
Enterohaemorrhagic *E. coli* (EHEC)	O26, O103, O111, O121, O145, O157	Watery or bloody diarrhoea, haemorrhagic colitis progression to systemic diseases	T3SS, Shiga-like toxins, enterohaemolysin, plasmid encoded protease (EspP)
Enteroaggregative *E. coli* (EAggEC)	O3, O15, O44, O86, O111, O125	Persistent mucoid diarrhoea	Aggregative adherence, dependent on plasmid-encoded genes such as AAF-fimbriae. Also heat stable enterotoxin (EASTI) and plasmid-encoded enterotoxin (PET)
Enteroinvasive *E. coli* (EIEC)	O28ac, O29, O112ac, O115, O121, O124, O135, O136, O143, O144, O152, O159, O164, O167, O173	Watery or bloody diarrhoea	Cellular invasion similar to *Shigella* spp. dependent on proteins encoded on pInv plasmid, including a T3SS
Diffusely adherent *E. coli* (DAEC)	O126:H27	Watery diarrhoea	Diffuse adherence F1845 fimbriae, AIDA-I adhesin

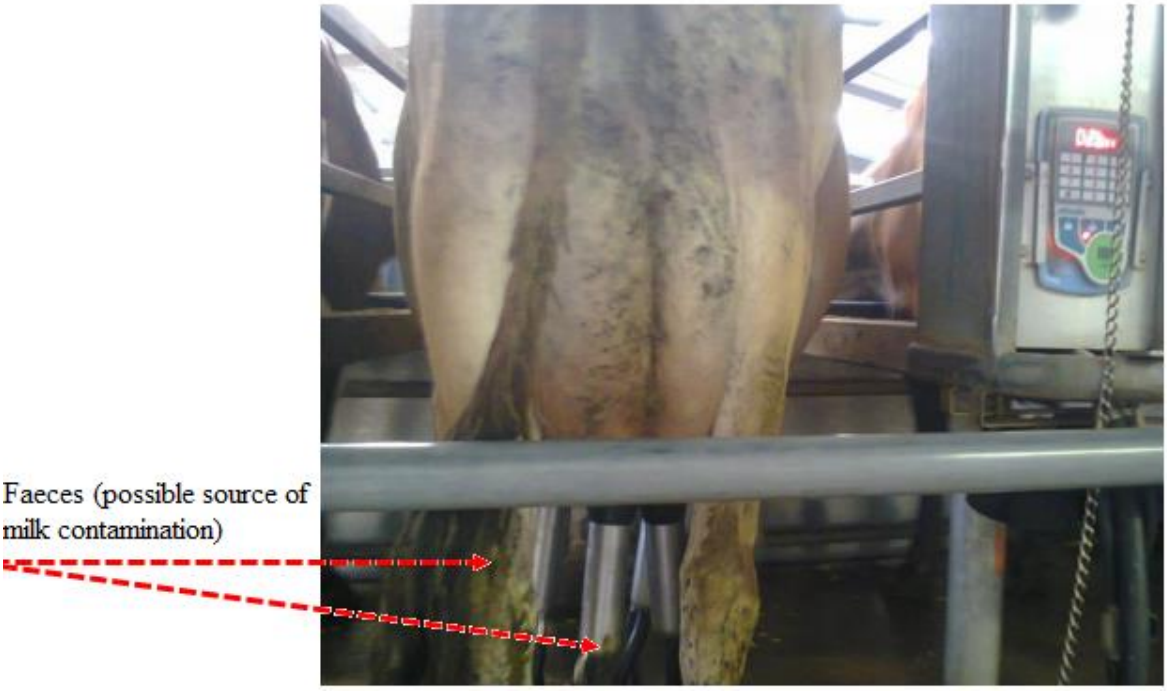

Figure 2.7: Cattle udder and back limbs covered with faeces during a milking process at a commercial dairy farm in the Eastern Cape Province, South Africa (Msilo, 2016).

Long-term sequelae of STEC infections range from mild diarrhoea and intestinal discomfort to serious complications, such as HUS and thrombotic thrombocytopenic purpura (TTP). Most documented outbreaks were linked to STEC O157 serotype, although other EHEC serotypes (non-O157) may also induce a range of other illnesses. Non-O157 STEC illnesses range from mild gastroenteritis to critical illness and death, either as sporadic cases or in outbreaks in certain geographical areas across the globe (Constantiniu, 2002). The lessened recognition and reporting of these non-O157 STEC infections serotypes globally can be attributed to inadequate analytical methods which also include lack of epidemiological and laboratory surveillance (as articulated in section 2.5.2.1). An estimate of over 265 000 illnesses in the USA are caused by STEC serotypes with 96.534 of those associated with STEC O157 and 168.698 non-O157 resulting in more than 3600 hospitalisations and 30 deaths each year (CDC, 2008). EFSA (2015) reports that, each year,

thousands of people are hospitalised due to complications linked to HUS. Nonetheless, in Africa, outbreaks associated with STEC in food and water are underreported, although a recent review (Raji et al., 2006) linked several outbreaks of STEC to food in the region. The first reported outbreak of HUS in Africa was from SA in the 90s and the transmission of the disease was argued to be contamination of surface water by dead and dying cattle, or consumption of meat from there, which was in turn exacerbated by a drought (Okeke, 2009). Several surveys in SA have detected STEC in beef, milk, faeces of food animals, as well as water used for irrigating fresh produce (Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2011; Aijuka et al., 2014; Caine et al., 2014; Iweriebor et al., 2015; Msolo, 2016). The National Institute for Communicable Diseases (NICD) in SA published some of the EHEC outbreaks in different provinces of the country (Table 2.7).

2.5.2.4. Pathogenicity of STEC

STEC is an insidious threat to food safety due to a number of virulence determinants, which are complex and multifactorial and these include shigatoxin (*stx* 1 and *stx* 2), intimin (*eae*), enterohaemolysin and the STEC autoagglutinating adhesion (*saa*). These virulence factors are either chromosomal or plasmid encoded and allow the organism to attach and colonise the bowel, invade tissues, and produce toxins that contribute to disease symptoms and disease progression (Gyles, 2007).

Table 2.7: Annual reports on *Escherichia coli* (EHEC) surveillance in South Africa from 2011 to 2013 (National Institute for Communicable Diseases, SA).

Year	Months	Number of cases by age group (0 to 9 years)	Number of cases per-month	Region	Number of cases by region
2011	January	2	1	Gauteng	2
	April		1	Gauteng	
2012	February	2	1	Gauteng	2
	November		2		
2013	July	3	1	Gauteng	2
	November		1	Mpumalanga	1

Shigatoxin virulence factors

Shigatoxins have played a major role in the severity of STEC induced diseases. Stx is a potent cytotoxin that damages the intestinal mucosal vasculature during haemorrhagic colitis and causes damage to the renal, circulatory and central nervous system, leading to HUS and TTP. Stx family consists of Stx 1 and Stx 2, which are related in structure and have similar biological activity. Stx 1 is very similar to type 1 toxin of *S. dysenteriae;* only differing in a single amino acid, while Stx 2 is genetically and immunologically, with less than 60% amino acid, homologous to Stx 1 (Melton-Celsa et al., 2011). *Stx 1* gene is highly conserved with little sequence variation but *Stx 2* has several subtypes, which differ in biological activity and immunological reactivity (Kaper, 1998; Scheutz et al., 2012). The phage-encoded toxin belongs to the AB_5 family of the toxins and consists of a pentameric ring-shaped B subunit (five 7.7kDa monomers) that is non-covalently attached to the catalytic A subunit (32kDa) (Fraser et al., 2004; Jafari et al., 2012). The B subunit interacts with globotriaosylceramides (Gb3) (also known as CD77) on the surface of human

intestinal mucosa and kidney epithelial cells resulting in internalisation of the toxin by endocytosis, in clathrin coated pits. The toxin is transported to the cytoplasm via the endoplasmic reticulum and Golgi apparatus, followed by the cleavage of the A1 peptide of the A-subunit by furin, releasing a 28kDa catalytically active protein, which acts to cleave 28S rRNA, resulting in the inhibition of protein synthesis and apoptosis (Kaper, 1998; Fraser et al., 2004). Host inflammatory response to STEC has been demonstrated to enhance the effects of Stx as TNFα and IL-1β levels increase the presentation of Gb3 receptors on human vascular endothelial cells (Melton-Celsa et al., 2011). Furthermore, the host inflammatory response releases polymorphonuclear leukocytes (PMLs), which are distributed to different target organs as a result of STEC infections. This transports Stx as well to these organs as the toxin has been observed to bind to the surface of these cells (Clark, 2009).

Stx 2 has been linked to increased likelihoods of causing HUS. There are 11 variants of Stx 2 cytotoxin (Stx2EDL933 (also termed Stx2vha), Stx2c (also termed Stx2vhb), Stx2d, Stx2e, Stx2f and Stx2g) which are antigenically non-cross reactive (Fraser et al., 2004). Levels of toxicity of Stx vary with Stx subtypes. The Stx2c has been isolated more frequently from HUS patients but Stx2e and Stx2f have been mainly isolated from pigs and birds and rarely from humans (Melton-Celsa et al., 2011). Toxicity of activated Stx2d is greater than that of Stx2c and the activity of Stx 1 and Stx2e are the least verotoxic (Clark, 2009). A study carried out in Australia discovered a different AB_5 toxin (subtilase-like toxin (SubAB)) in EHEC which differs significantly from other AB_5 toxins (Beddoe et al., 2010). The SubAB shows greater cytotoxicity than Stx 2 for a range of cell types, including Vero cells. STEC can release the Stx in different foods, which include milk and ingestion of such food poses a risk. Once the Stx protein is released in the food it is not inactivated by pasteurisation temperatures (Melton-Celsa et al., 2011).

Adherence factors (attaching and effacing (A/E))

There is a complex interplay between EHEC virulence factors during intestinal colonisation. The EHEC genome, in addition to *Stx* genes, contains a pathogenicity island known as the locus of enterocyte effacement (LEE) (Deng et al., 2004). The locus encodes the genes necessary for STEC to induce attaching/effacing (A/E) lesions associated with EHEC, including a functional type III secretion system (T3SS) (Perna et al., 1998; Clark, 2009). These systems include structural components of a type III secretion system (TTSS), intimin (encoded by the *eae* gene), and translocated intimin receptor (Tir) and other effector proteins (Kaper, 1998; Deborah Chen and Frankel, 2005). The LEE (approximately 35.5kbp) is organised into 5 operons (Elliott et al., 1998): (i) Operons 1-3 encoded by the *esc* genes that secretes the proteins required to form the secretion apparatus. (ii) Once the secretion apparatus is formed, EspA, which is encoded by the LEE4 operon secretes protein A. Protein A forms the needle structure of the T3SS, EspB and EspD, which form a pore in the host cell membrane (Perna et al., 1998; Clark, 2009). (iii) Once the lesions are formed, a number of proteins are injected into the cell, which include translocated intimin receptor (Tir) encoded by the LEE5 operon. The Tir becomes embedded in the host cell membrane and interacts with intimin (also LEE5-encoded). Intimin is a bacterial outer membrane protein and this interaction facilitates intimate adherence between the bacterium and the host (Elliott et al., 1998). Figure 2.9 depicts the LEE structure in EHEC.

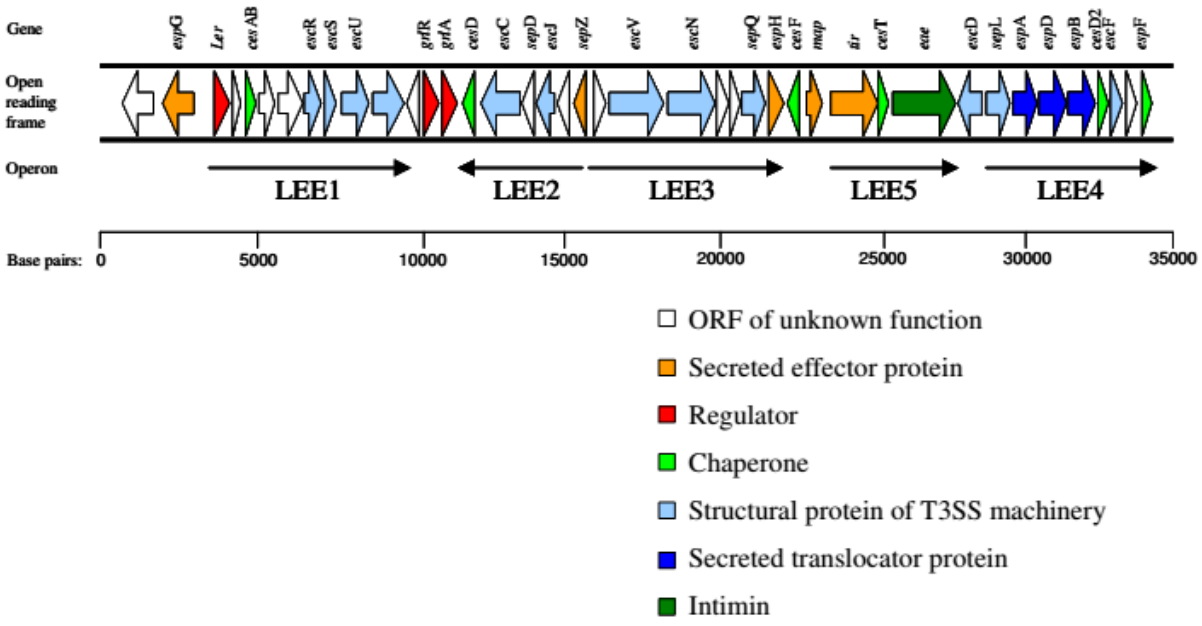

Figure 2.8: Organisation of genes broadly conserved between the LEE of EHEC (Clark, 2009).

STEC containing LEE attach to the host intestinal mucosa and destroy the surrounding microvillus brush border, which causes substantial cytoskeletal rearrangements within the enterocyte (Kaper et al., 2004). This ability produces A/E lesions, which are sufficient to cause non-bloody diarrhea although Stx is essential for the development of bloody diarrhea, HC and HUS. Stx is absorbed into the host cell through a transcellular pathway after the attachment (Nataro and Kaper, 1998). STEC uses other adherence factors apart from intimin for pathogenesis as evidenced by some non-O157 isolated from outbreaks which lacked the *eae* gene (Nataro and Kaper, 1998; Paton et al., 2001). However, none have been well characterised or specifically demonstrated to play a role in adherence in vivo (Elliott et al., 1998). The *saa* is a new virulence gene which is believed to work as an adherence factor in *eae* negative strains (Paton et al., 2001). Other factors which were reviewed and suggested to have potential in facilitating adherence in STEC are 94-kDa OMP

protein, lipopolysaccharides, Type 1 fimbriae and a protein with homology to the iron-regulated gene A (*irgA*) of *V. cholera.*

Other potential virulence factors

Some STEC/EHEC isolates have been shown to possess the enteroaggregative heat-stable enterotoxin1 (EAST1) protein and usually two copies of the *ast*A gene is present in the chromosome (Savarino et al., 1996). EASTI exhibits some functional relatedness to heat-stable enterotoxin a (STa) of ETEC, although it is distinct from other *E. coli* heat-stable toxins. Some of the non-bloody diarrhea (mild and watery diarrhea) in a person infected with these strains might be due to the production of this toxin (Savarino et al., 1996) .

Most of the virulence determinants of EHEC strains are chromosomally encoded; nonetheless, plasmids might also play an important role in its pathogenesis. Some of the STEC serotypes (99 – 100% of O157:H7) possess highly conserved plasmids which vary in size from 90 to 104 kilobase such as pO157, pSFO157 and pO113 (Nataro and Kaper, 1998). The plasmids have been correlated with haemolytic activity and adherence to intestinal epithelial cells, although without substantiating evidence (Mahajan et al., 2009). The most isolated plasmid in STEC, following outbreaks, is the pO157 (Abu-Ali et al., 2010a). Large intact plasmid or expression of high levels of plasmid encoded products has been detected more often associated with HUS than those without pO157 (Abu-Ali et al., 2010b).

The flagella are filamentous appendages, which the bacterium may rotate to facilitate motility. Flagella and T3SS share structural and functional similarities and the polymerisation of flagellin is proposed to occur by a similar mechanism to the translocation of T3SS effector proteins (Clark, 2009). Recent evidence suggests the facilitation of the initial localisation of the bacterium to the

mucosal surface and the subsequent binding to intestinal mucins by the H7 flagellin in STEC/EHEC (Erdem et al., 2007; Mahajan et al., 2009).

Another important plasmid encoded factor is the enterohaemolysin proteins. The 60-MDa plasmid is found in all O157:H7 strains but not in all Stx producing *E. coli* (Abu-Ali et al., 2010b). Some non-O157 (O111:H7) serotypes isolated from disease outbreaks did not contain the enterohaemolysin genes (Nataro and Kaper, 1998). The genes for enterohaemolysin include *ehxA, hlyA, sheA,* and *e-hlyA,* which codes for proteins responsible for cell lysis whereby the membranes of target cells including erythrocytes, leukocytes and renal tubular cells are perforated (Kerényi et al., 2005).

2.5.2.5. Treatment of STEC infections and its control

STEC infections vary within the subgroup of the population although most people tend to recover within 5 to 7 days of infection (Msolo, 2016). Suggested treatment for STEC infection includes: (i) The use of antibiotics. However, the use of antibiotics in treating STEC infections is heavily discouraged due to lack of evidence that substantiates that treatment with antibiotics is helpful. Furthermore, the use of these antibiotics, such as quinolone has been argued to increase the risk of HUS (Muniesa et al., 2012). (ii) Once STEC infection develops to HUS, it is very difficult to treat, therefore, kidney dialysis, blood transfusion and/or plasma therapy and renal transplantation have been employed (Muniesa et al., 2012). Literature has also suggested re-hydration of infected individuals to avoid dehydration and to seek medical attention immediately (Msolo, 2016).

STEC infections can be prevented by: (i) General personal hygiene, consuming pasteurised milk and state certified raw milk and its products, sufficient heat treatment of food before consumption (Menrath et al., 2010). (ii) Prevention and control of infection by targeting environmental and

animal sources of the organism. Reducing the STEC from the vector (cattle) may in turn help to reduce incidences of milk and other foods of bovine origin linked outbreaks (Clark, 2009). Interventions include animal and equipment cleanliness, improving feeding and housing strategies, clean rinsing water for milking machine and equipment washing, not milking animals with mastitis and ensuring a cold chain during milk transportation and storage (Van Kessel et al., 2004). It is also important to prevent the contact between wildlife and cattle as wildlife has been reported to transmit EHEC to cattle (García et al., 2010). Other reviewed intervention methods include: modulation of gastrointestinal acidity by switching feed from grain to hay prior to slaughter, control methods employing antimicrobial substances, vaccination strategies, biological control using bacteriophages and use of probiotic bacteria in animal feed (Clark, 2009).

2.5.2.6. STEC O157 and non-O157 in dairy

Several serotypes of STEC have been linked to foodborne illnesses associated with the consumption of milk and dairy products. However, it is difficult to estimate the true percentage of non-O157 outbreaks and infections associated with milk and dairy since they are not routinely subjected to testing (Mathusa et al., 2010). In the USA, the active surveillance of foodborne illnesses and infections attributed to non-O157 serotypes began in late 2001 (Mathusa et al., 2010). In Europe, more than half of the reported cases associated with dairy outbreaks in 2009 were ascribed to the O157 serogroup and 5.4% to the O26 serogroup (EFSA, 2011). The most implicated non-O157 serogroups linked to foodborne outbreaks associated with dairy in the USA, EU, Canada and Japan are O26, O103, O91, O145 and O111 (EFSA, 2015). Except for O91, these serogroups also accounted for the majority of non-O157 serogroups associated with HUS (Farrokh et al., 2013). As a result of lack of investigation and also to lower pathogenicity, the number of reported outbreaks due to non-O157 STEC remains relatively low. Table 2.5 shows the STEC

outbreaks that have been linked to milk and dairy products. Dairy products implicated in outbreaks associated with STEC serotype include soft and semi-soft cheeses, yoghurt, farm ice-cream and milk shakes made from raw and pasteurised milk (Honish et al., 2005; Mathusa et al., 2010). Dairy products made from pasteurised milk have also been reported in milkborne outbreaks linked to STEC serotypes (O145:H28 and O26:H11), although these were probably due to defective pasteurisation and/or post processing contamination (Baylis, 2009).

2.6. ANTIBIOTIC RESISTANCE IN MILKBORNE MICROORGANISMS

The introduction of antibiotics in the 1930s was life changing in terms of improving human and animal health as they were used to treat infectious diseases of humans and animals (Oliver et al., 2011). However, despite these benefits, there is considerable concern from the public health, food safety, and regulatory perspectives about the use of antimicrobials in food-producing animals (Oliver et al., 2011). Antimicrobials are administered for both therapeutic and prophylactic purposes in food animals and dairy cattle. Currently, there is a growing global concern over the development of antimicrobial resistant bacteria resulting from the agricultural use of antibiotics that could impact treatment of diseases affecting the human population that require antibiotic intervention. Conversely, other scholars have disputed this notion (Oliver et al., 2011; da Silva and Mendonça, 2012). Over the years, several reviews have been published on topics of antimicrobial use in dairy animals and development of antimicrobial resistance in milkborne microorganisms (Khachatourians, 1998; Oliver et al., 2011; da Silva and Mendonça, 2012; Landers et al., 2012; Ruegg, 2013). Factors giving rise to antimicrobial resistance in milk borne microorganisms are not clear, although, the inherent consequence of exposure to antibiotic compounds can give rise to resistance as a result of natural selection (Aminov and Mackie, 2007). The use of antimicrobials at subtherapeutic concentrations in dairy animal production systems

could result in the development of antimicrobial resistance in commensal and pathogenic bacteria owing to selection pressure, and favor genetic exchange of antimicrobial resistance determinants involving commensal bacteria (Straley et al., 2006). Bacteria have been reported to exchange antimicrobial resistance genes, and these genes may ultimately enter human pathogenic bacteria through the food supply chain (Sayah et al., 2005). Antibiotic resistant STEC, *Salmonella* spp., and *L. monocytogenes* have been isolated from milk and dairy products (Oliver et al., 2005). Recent reports from the CDC have revealed an unprecedented outbreak associated with antibiotic resistant *Salmonella* in food (Ventola, 2015).

2.6.1. Important types/classes of resistance

Each antibiotic operates at a specific site within the bacterial cell which include: (i) interference with cell wall synthesis (e.g. beta-lactams and glycopeptide agents), (ii) inhibition of protein synthesis (e.g. macrolides and tetracyclines), (iii) interference with nucleic acid synthesis (e.g. fluoroquinolones and rifampin), (iv) inhibition of a metabolic pathway (e.g. trimethoprim-sulfamethoxazole), and (v) disruption of bacterial membrane structure (e.g. polymyxins and daptomycin) (Tenover, 2006). While bacteria resistance may be specific to particular antibiotics or intrinsically resistant to a class of antimicrobial agents, the same resistance mechanism can be used to resist several antimicrobial agents, even from different classes. Mechanisms of how bacteria can acquire resistance are either by *de novo* mutation or via the acquisition of resistance genes from other organisms through horizontal gene transfer (HGT) (Ventola, 2015). HGT by antimicrobial-susceptible bacteria from resistant strains may occur through conjugation, transformation or transduction, with transposons often facilitating the incorporation of the multiple resistance genes into the host genome or plasmids (Tenover, 2006). These transposons (which include plasmids) can carry virulence factors, which also equip the recipient with virulence genes.

A review by da Silva and Mendonça (2012) revealed an association between antibiotic resistance and virulence factors in *E. coli* as a result of lateral gene transfer.

Some Gram-negative bacteria belonging to the Enterobacteriaceae family (which include *E. coli*) have been reported to produce β-lactamases to resist cephalosporins and aztreonam. Resistance to cefpodoxime has been reported to be mediated by a mutation in the attenuator or the weak promoter of the chromosomal gene, *ampC β-lactamase,* and this leads to enhanced production of AmpC, resulting in resistance to extended-spectrum cephalosporins (Caroff et al., 1999). These enzymes cleave the amide bond in the β-lactam ring, rendering β-lactam antibiotics harmless to bacteria. The β-lactamase enzymes differ from one bacterium to another and they are classified into four classes, designated A to D, on the basis of their amino acid sequences. The most frequent class is A and C. Overuse of oxyimino-cephalosporins such as cefotaxime and ceftazidime induce the appearance of resistant strains, which overproduce class C enzymes and/or which produce extended spectrum β-lactamases (ESBLs), mainly those of class A but also those of class D (Bonnet, 2004). Class A ESBLs hydrolyze oxyimino-cephalosporins and aztreonam, although they are generally susceptible to β-lactamase inhibitors (clavulanate, sulbactam, tazobactam) (Bonnet, 2004). Class A, used to differ from widespread plasmid-mediated TEM-1/2 and SHV-1 penicillinases by one to four point mutations, which extend their hydrolytic spectra. However, TEM and SVH ESBLs now harboured by at least 130 members including Enterobacteriaceae species (Caroff et al., 1999). Currently, ceftazidimases of the PER, VEB, TLA-1, and GES/IBC types and cefotaximases of the SFO-1, BES-1 and CTX-M types have been reported and the CTX-M β -lactamases are the most widespread enzymes (Bonnet, 2004).

2.6.2. Occurrence of antibiotic resistant *E. coli* in dairy

Some studies have isolated antibiotic resistant STEC in milk and dairy products (Solomakos et al., 2009; Momtaz et al., 2012). Very few or no studies have isolated Enterobacteriaceae producing ESBLs in milk. The reason for antibiotic resistant bacteria in milk can be the overuse of drugs in agriculture as alluded to in section 2.6. Few studies have justified this notion. Nonetheless, a longitudinal study of *E. coli* O157:H7 dissemination related the occurrence of antibiotic-resistant isolates on Wisconsin dairy farms in the USA to the therapeutic and subtherapeutic (in feed) use of antibiotics (Khachatourians, 1998). The same study reports that over a 14-month period, subtherapeutic use of antibiotics (penicillin, sulfamethazine, chlorotetracycline, oxytetracycline and neomycin, 0.25 to 1 kg per ton of feed or added to drinking water) and therapeutic use of sulfamethazine to treat diarrhea in dairy cattle, correlated well with the emergence of antibiotic-resistant *E. coli* O157:H7. In SA, limited studies have isolated drug resistant *E. coli* in dairy (Msilo, 2016).

2.7. MICROBIAL RISK ASSESSMENT AND POTENTIAL ROLE IN MILK SAFETY

The management of food safety is changing on a global level to meet the challenge of changing patterns in food trade, such as globalisation of the food supply (Brown, 2002). Food safety hazards may enter at various stages along a dairy supply chain. Several intervention measures, such as the implementation of GLOBAL Good Agricultural Practices (GLOBALG.A.P.) or Hazard Analysis Critical Control Points (HACCP) systems, have been implemented to control hazard presence in dairy (Asselt et al., 2017). Milk and dairy outbreaks are still reported regardless of these quality control programs being in place. Therefore, monitoring programs have been established to detect the possible presence of food safety hazards (Noordhuizen and Metz, 2005) and these should be

risk-based and focus on the most relevant food safety hazards. Risk based approaches (risk analysis) offer a new means of improving and managing food safety associated with, especially, pathogenic microorganisms and other chemical hazards (Grace et al., 2010). Risk analysis is composed of three components, which are risk assessment, risk management and risk communication. Figure 2.10 represents the interaction of these three elements. Voysey and Brown (2000) defined: (i) Risk assessment as the measurement of risk and the identification of factors that influence it, (ii) Risk management as the development and implementation of strategies to control the risk and (iii) Risk communication as the exchange of information relevant to the risk among interested parties.

The first stage in risk analysis is to conduct a risk assessment. Successful completion of this stage allows decision makers to set an appropriate level of protection, based on evidence rather than anecdote and subjective preferences. Risk assessment studies have been carried out to quantify the risk posed by pathogenic organisms in food, which include milk (Duffy et al., 2006; Delignette-Muller et al., 2008; Grace et al., 2008; Latorre et al., 2011; EFSA, 2015; Giacometti et al., 2015; 2016), at national and regional levels by different researchers across the globe. To-date, quantitative microbial risk assessment (QMRA) for the major milk borne pathogens have been conducted. EFSA (2015) and Giacometti et al. (2012a; 2015; 2016) conducted risk assessment for *L. monocytogenes*, *C. jejuni*, STEC O157 and *Salmonella* spp. in raw drinking milk in Europe. The risk of listeriosis associated with the consumption of milk was also evaluated in the USA (CDC, 2010; Latorre et al., 2011). Few risk assessment studies on pathogen contaminated foods have been carried out in Africa. However, Grace et al. (2008) and Makita et al. (2010) estimated the risk of HUS and brucellosis incidence in informally marketed milk in Africa, respectively. Risk assessment studies carried out for different pathogenic microorganisms associated with milk in

different countries are presented in Table 2.8. The major shortcoming outlined by researchers who conducted QMRA in Africa was applying risk based methods to diverse, non-linear, shifting and data scarce systems in which formal and informal food supply systems co-exist and overlap.

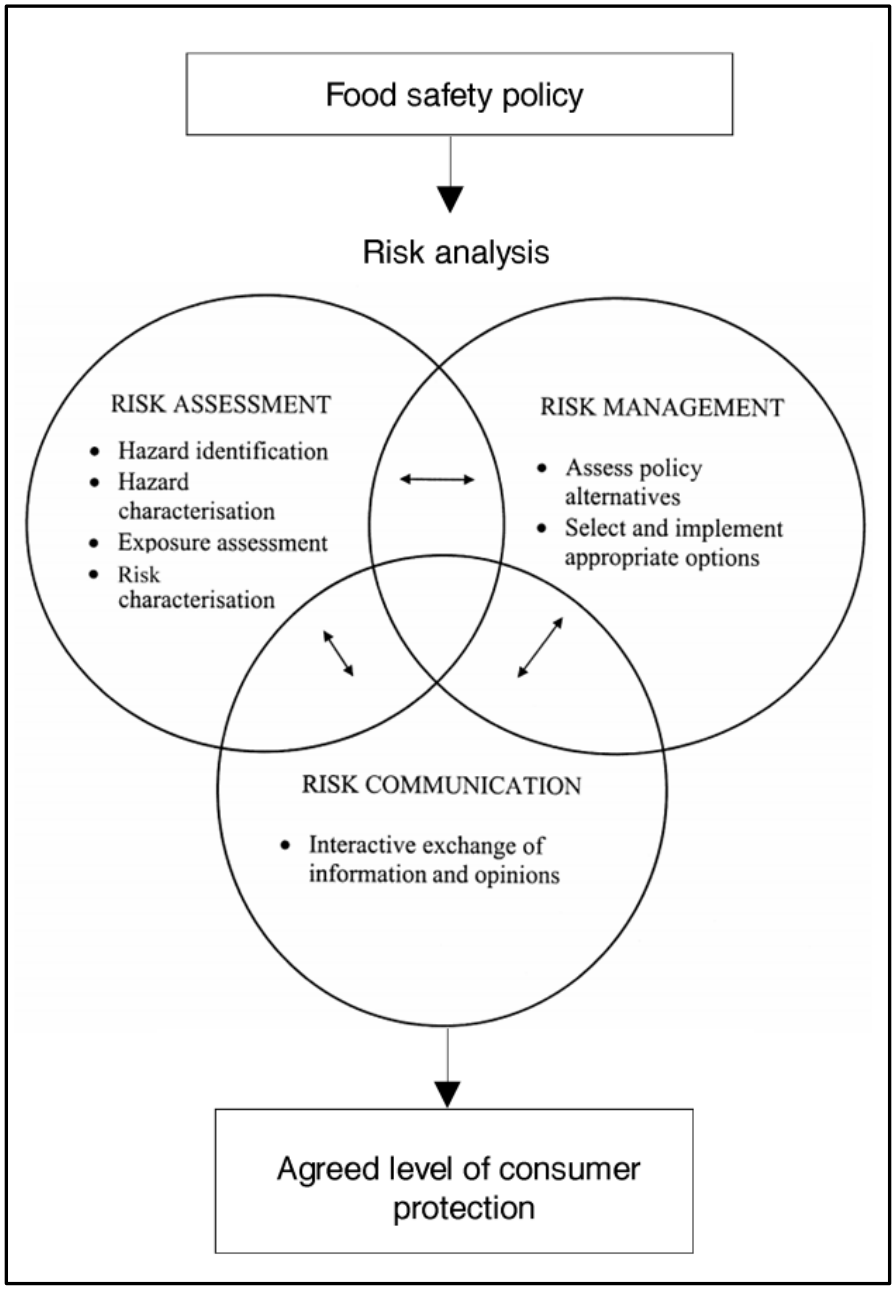

Figure 2.9: Risk analysis framework (Adapted from Lammerding, 1996).

Table 2.8: Risk assessment studies carried out for different pathogenic microorganisms associated with milk in different countries.

Country	Microbial hazard						Reference
	***Campylobacter* spp.**	**STEC**	***Staphylococcus aureus***	***Listeria monocytogenes***	***Brucella* spp.**	***Salmonella* spp.**	
Australia	✓	✓		✓	✓		(FSANZ, 2009)
USA			✓	✓			(Heidinger et al., 2009; Latorre et al., 2011)
Italy	✓	✓		✓		✓	(Giacometti et al., 2012a; Giacometti et al., 2015; Giacometti et al., 2016)
New Zealand	✓	✓		✓		✓	(Soboleva, 2013)
Uganda					✓		(Makita et al., 2010)
East Africa		✓					(Grace et al., 2008)
UK		✓					(Clough et al., 2009)
Sweden			✓				(Lindqvist et al., 2002)
France		✓		✓			(Bemrah et al., 1998; Perrin et al., 2015)

*(✓) = Represent the pathogenic microorganisms for which the risk assessment was conducted. STEC – Shigatoxin producing *E. coli.*

Microbial risk assessment is a valuable tool used to organise and analyse scientific information to estimate the probability and severity of any adverse risk posed by a pathogen in a particular food commodity (Codex Alimentarius Commission, 1999; Duffy et al., 2006). The ultimate goal is to reduce and prevent the risks presented by hazardous microorganisms, whether natural or anthropogenic, intentional or unintended. Risk assessment can be conducted qualitatively or quantitatively and it includes four stages (Figure 2.11): (i) hazard identification, (ii) exposure assessment, (iii) hazard characterisation and (iv) risk characterisation as outlined by the Codex Alimentarius Commission (Codex, 1999).

2.7.1. Hazard identification

Hazard identification is conventionally the first step in risk assessment. A hazard is an agent having an adverse effect on the public health of the human population and may pose a short term, chronic, or fatal risk to a person (Voysey and Brown, 2000). Hazard identification recognises the causal relationship between a pathogenic agent, an illness and a food as one vector of a specified illness (Brown, 2002). Information about a microbial hazard associated with a particular food is obtained from routine microbial analysis of the commodity or from an epidemiological data (Voysey and Brown, 2000). Hazard identification in qualitative risk assessments involves gathering and collating existing information on the characteristics of the pathogen that affect its ability to be transmitted by the product and to cause disease in the host, whereas, in quantitative risk assessment, this information serves as an input to the development of the model for exposure assessment and dose-response (Brown, 2002).

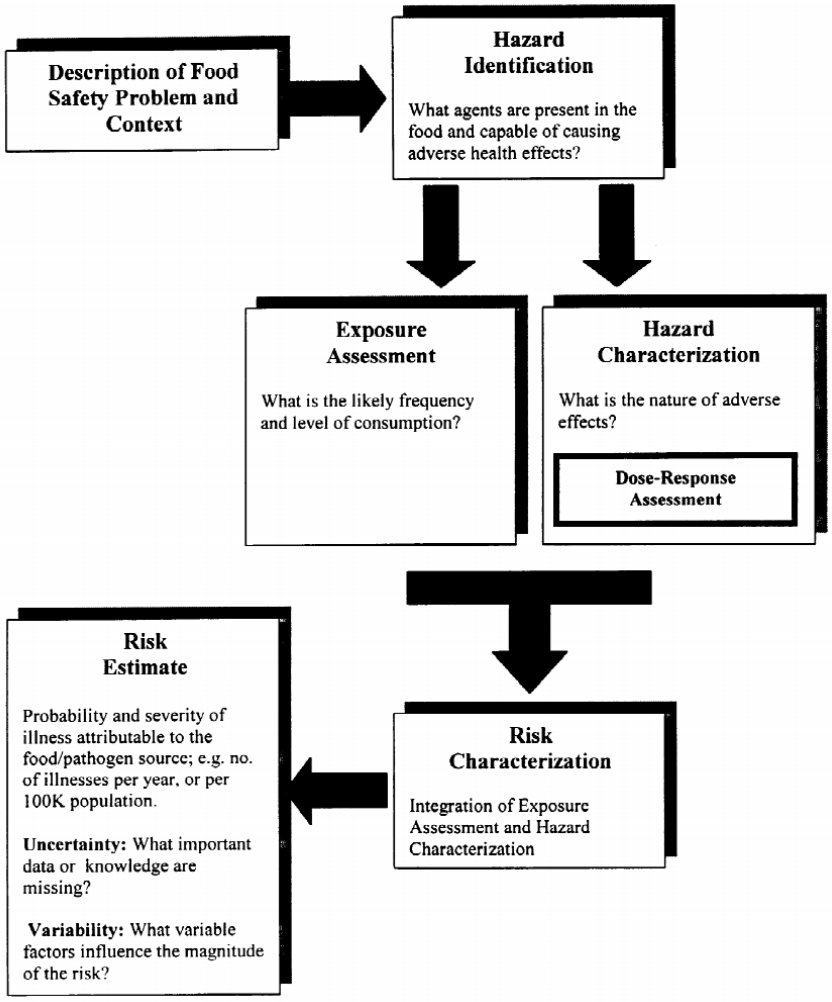

Figure 2.10: Codex Committee on Food Hygiene Principles and Guidelines for the Conduct of Microbiological Risk Assessment (Codex Alimentarius Commission, 1999).

Numerous foodborne hazards have been identified and documented (Meng and Doyle, 2002). The leading microbiological hazards of public health concern causing periodic milkborne outbreaks are presented in Table 2.4. Emerging foodborne pathogens such as non-O157 STEC in food such as milk are receiving attention as hazards of concern by stakeholders. Lately, milkborne disease outbreaks were incriminated to the consumption of raw milk contaminated with STEC in the EU and USA, especially raw milk sold directly from producer to the public (CDC, 2007; Denny et al., 2008; EFSA, 2015). A review by Claeys et al. (2013) reported 13 *E. coli* outbreaks in Europe and 28 worldwide associated with the consumption of raw milk, between 1970 and 2010. The same authors document an increased incident consisting of 27 STEC outbreaks in Europe between 2007 and 2012 as a result of raw milk consumption. However, few outbreaks were reported for pasteurised milk during that period (Clough et al., 2009). Farrokh et al. (2013) document STEC outbreaks from 1986 to 2010 that have been linked to milk and dairy products in Europe, USA and Canada. Most of these outbreaks, reviewed by Farrokh et al. (2013), were associated with STEC O157, although other serotypes or serogroups, including O22:H8, O110:H^{-}, O80:H^{-}, and O145 have also been identified as causative agents.

2.7.2. Hazard characterisation

In the Codex framework, hazard characterisation provides a qualitative and/or quantitative evaluation of the hazard with the probability of illness or death (Voysey and Brown, 2000; Brown, 2002; Duffy et al., 2006). This involves a dose–response relationship, which combines subsequently with the potential for exposure, to estimate the amount (number) of pathogens which cause illnesses. Different subpopulations (children, elderly, males, females and the immunocompromised) respond variably to a pathogen. The frequency, severity and duration of a microbiological disease is dependent on a variety of interacting factors related to the pathogen,

such as the host's susceptibility (immune status and immunity imparted by previous exposure), dose ingested and the environment, including the food vehicle (Brown, 2000). Dose–response models are difficult to develop due to ethical issues. Nonetheless, they can be derived from human clinical trials, epidemiological studies based on food outbreaks, animal clinical trials, in vitro studies using cell lines, biomarkers or expert opinion (Duffy et al., 2006). Several dose–responses that describe the susceptibility to different food and water-borne pathogens of different sub populations are available (Teunis et al., 1999; Buchanan et al., 2000). Table 2.9 depicts the mathematical models that have been used to empirically describe dose-response data for foodborne pathogenic bacteria. For pathogenic *E. coli* most research studies have used the exponential dose-response model, were *r* values are derived for each subpopulation.

2.7.3. Exposure assessment

According to the codex definition, exposure assessment is a quantitative estimation of the presence of a contaminant in a serving of food at the time of consumption. However, the description of exposure may be more broadly understood, and include the accumulation of data on the prevalence and concentration of a pathogen at key points in the food chain. This also includes data on how particular stages in the food chain affect the numbers/prevalence of the pathogen and the characterisation of the nature and size of the population (or subpopulations) exposed, and of the route(s), magnitude, frequency and/or duration of that exposure. Transmission of the pathogen involved is modelled throughout the food pathway i.e. from 'farm to fork'. According to Nauta (2002), this transmission model follows probability distributions of the prevalence and concentration of the pathogen along the food pathway. The 'farm to fork' pathway approach predicts the identification of risk mitigation points along the food chain (Nauta, 2002; Grace et al., 2010).

Table 2.9: Mathematical models that have been used to empirically describe dose-response data for foodborne pathogenic bacteria (Buchanan et al., 2000).

Model	**Formula**
Exponential	$P(d) = 1 - \exp(-rd)$ where: $P(d)$ = probability of infection at dose (d) d = dose (CFU) r = model parameter specific for each pathogen
Beta-Poisson	$P(d) = 1 - (1 + d/\beta)^{-\alpha}$ where: $P(d)$ = probability of infection at dose (d) d = dose (CFU) α = model (infectivity) parameter β = model (shape) parameter
Weibull-Gamma	$P(d) = 1 - [1 + (d^{b}) / \beta]^{-\alpha}$ where: $P(d)$ = probability of infection at dose (d) d = dose (CFU) b = model (shape) parameter α = model (infectivity) parameter β = model (infectivity) parameter
Weibull	$P(d) = 1 - \exp(-ad^{b})$ where: $P(d)$ = probability of infection at dose (d) d = dose (CFU) a = model (infectivity) parameter b = model (shape) parameter
Gompertz	$P(d) = 1 - \exp[-\exp(a + bf(d))]$ where: $P(d)$ = probability of infection at dose (d) d = dose (CFU) a = model (intercept) parameter b = model (slope) parameter $f(x)$ = function of dose

Albeit several exposure assessment approaches being suggested for 'farm to fork', the widely used is the modular process risk model (MPRM) frame work. Most published risk assessment studies for pathogens in frozen ground beef (Delignette-Muller et al., 2008; Signorini and Tarabla, 2009), vegetables (Hamilton et al., 2006) and milk (Latorre et al., 2011) have used the MPRM

framework. The description of the MPRM framework and associated predictive models are outlined in publications by Nauta (2002) and Brul et al. (2007). The first stage in the MPRM is to estimate the initial concentration of pathogen in the food. Very few studies have quantified pathogen levels in food. Using prevalence data of the pathogen in food samples, concentration can be estimated using Bayes' theorem techniques (Giacometti et al., 2012a; Giacometti et al., 2015). The final step in the process estimates the amount/concentration of contaminant in a single serving of food (Duffy et al., 2006). Exposure assessment models combine both deterministic (derived using single data points along the food chain) or probabilistic (in which a distribution curve representing all data is used as opposed to a single point estimate) modelling.

A common approach is the probabilistic modelling, which gives a more precise estimation of the risk than the deterministic, which overestimates or underestimates the risk (Duffy et al., 2006). With the advent of technology, new software such as @Risk (Palisade, NY, USA) and Crystal Ball (Decisioneering Inc., Denver, USA) are used to conduct probabilistic exposure assessments using Monte Carlo simulations. These recent software are also capable of performing sensitivity analysis, which determines the effect of model parameters on output (risk of illness/death). Most risk assessments for different pathogens in milk have noted initial concentration and prevalence of a pathogen in milk at the farm, milk storage temperatures and handling practices at house hosts as the most important parameters, which increase the risk (Latorre et al., 2011; Geser et al., 2012; Giacometti et al., 2015). Exposure assessment is also characterised by uncertainty and variability in data inputs and this should be clearly identified and taken into account by decision makers (Nauta, 2007).

2.7.4. Risk characterisation

The final stage in the process estimates the adverse public health effect or risk as a consequence of the integration of the exposure and dose–response assessments. Risk characterisation can be a prediction of illness per typical serving, number of portions consumed per unit time, calculated as an annual risk of illness/death per subpopulation (Voysey and Brown, 2000; Duffy et al., 2006). Depending on the hazard characterisation data available, the overall probability of occurrence and severity of health effects in a given population may be broken down into age categories, based on differences in immune status in order to identify groups which may be at higher risk following exposure to the contaminant (Duffy et al., 2006). Commercial software are used in risk characterisation and to allow a more complex risk estimation and analyses of the data, the risk characterisation should include a description of statistical and biological uncertainties and variability using the software.

At this level, if not at the exposure assessment stage, sensitivity analysis can also be performed to assess, which parts of the chain significantly affect risk or to assess the changes in predicted illness by incorporating a new hypothetical risk mitigation strategy at a particular point in the chain (Voysey and Brown, 2000; Duffy et al., 2006). An estimate of 2,402 to 2,835 cases of symptomatic VTEC infections per 10,000 servings were reported in a risk assessment carried out for STEC in unpasteurised milk (Grace et al., 2008). Giacometti et al. (2012a) report that for 5.25 million milk portions consumed under the worst and best storage conditions, the expected numbers of HUS cases per year were 0.09 and 0.02, respectively for the 0- to 5-year age group; and 0.5 and 0.1, respectively, for the >5-year age group. The same authors also conducted a risk assessment on paediatric HUS cases related to the consumption of raw milk sold through vending machines in Italy. They observed that the total number of HUS cases (associated to raw milk) was a function

of the percentage of consumers drinking raw milk. For example, in the worst case scenario, the average HUS probability per serving was 8.25×10^{-7} in the case of 1% of consumers drinking raw milk and the probability rose to 1.87×10^{-5} in the case of 20% of consumers drinking raw milk (Giacometti et al., 2016).

2.8. CONCLUSIONS AND KNOWLEDGE GAPS

PDBM plays an important role in the diet of people in SA and across the globe, supporting their livelihoods. This review established that the production, processing and distribution of PDBM can lead to contamination by spoilage and pathogenic microbiota which include *E. coli* and its pathogens. Therefore, contaminated PDBM is a potential public health risk. Studies in SA have evaluated the quality of PDBM sold directly from the producer to the consumer, but there are no studies which actually characterise spoilage and pathogenic microorganisms in the milk. The review reveals that STEC is one of the major pathogens of public health significance, causing outbreaks associated with milk and dairy around the world. The most widely used analytical method for detecting STEC in milk only aims at detecting *E. coli* O157 because the techniques are available and established, and there are relatively few investigations aimed at detecting other STEC serogroups. Subsequently, several research studies have characterised STEC O157 in milk but very few or none have investigated the presence of non-O157 STEC serotypes in PDBM, albeit non-O157 being linked to fatal illnesses in other geographical parts of the world. Furthermore, antimicrobial resistance in *E. coli* is of major public health concern, globally. *E. coli* is capable of producing ESBLs and resisting cephalosporins. Different studies have isolated ESBLs *E. coli* from dairy cattle and cattle faeces but there are no studies which have elucidated ESBLs Enterobacteriaceae in milk. Risk assessment for major pathogens, including STEC, in raw and pasteurised milk have been conducted in Europe, USA and in Africa. However, there are no risk

assessment studies conducted to estimate the burden of illness associated with the consumption of pathogen contaminated PDBM. Thus, it is important to investigate the presence of STEC O157 and non-O157 in PDBM, and to evaluate the risk associated with the consumption of such milk.

2.9. HYPOTHESES AND OBJECTIVES

2.9.1. Hypotheses

(a) PDBM from different regions in SA is contaminated with STEC, belonging to different serotypes. STEC isolates from different regions will exhibit different virulent capacity, antibiotic profiles and toxigenicity.

Scientific justification of the hypothesis

It has emerged from recent findings in SA at the University of Pretoria, Department of Food Science and other research studies that *E. coli* contaminants from irrigation water and lettuce, as well as from bulk milk, might belong to different serotypes compared to their European food counterparts (MSc thesis Matthew Aijuka, 2012, Caine et al., 2014). Extrinsic/environmental factors, such as temperatures, oxygen and humidity are characteristics of a particular geographical region, which can influence genotypic and phenotypic variations in microorganisms. Due to these environmental pressures, genotypic and phenotypic variation can then be invoked by mutations and horizontal transfer of genes encoding positively selected traits as virulence-associated factors among genetically distinct strains (Lawrence, 1999).

(b) Consumption of STEC contaminated PDBM will result in HUS cases and the subsequent burden of illness is dependent on (i) subpopulation groups (age, sex, immunocompromised and perinatal) and (ii) quantity and frequency of consumption.

Scientific justification of the hypothesis

Quantitative microbiological risk assessment and predictive modelling are important scientific tools, which provide evidence-based and transparent estimation of the risk of foodborne illnesses (Codex Alimentarius Commission, 1999; Duffy et al., 2006). Several risk assessment studies have been conducted in USA, Europe and Africa, in an attempt to quantify disease cases as a result of milkborne pathogens, (Grace et al., 2008; Clough et al., 2009; Giacometti et al., 2015). *E. coli* pathogenicity is dependent on host susceptibility (immune status and immunity imparted by previous exposure) and dose ingested and the most vulnerable members to the diseases are children under 5 years, the elderly and immune-compromised individuals. However, some STEC strains (O104 and O157 serotypes) have proven to cause severe illnesses even in healthy adults (Mellmann et al., 2011). Ingestion of 5 to 100 cells of EHEC can cause fatal illness in humans (Nguyen and Sperandio, 2012; Dean et al., 2013).

2.9.2. Objectives

(a) To characterise *E. coli* and other Enterobacteriaceae in raw and pasteurised PDBM in SA

(b) To investigate for virulence genes (*stx1*, *stx2* and *hlyA*), serotypes and extended-spectrum β-lactamase (ESBL) producing capacity in O157 and non-O157 *E. coli* , isolated from PDBM

(c) To estimate the haemolytic uraemia syndrome (HUS) risk associated with the consumption of STEC contaminated PDBM and estimate the resulting burden of illness that may be associated with the consumption of PDBM in SA

CHAPTER THREE

CHARACTERISATION OF *E. COLI* AND OTHER ENTEROBACTERIACEAE IN PRODUCER-DISTRIBUTOR BULK MILK

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3.1. ABSTRACT

The current study was undertaken to characterise *Escherichia coli* and other Enterobacteriaceae in raw and pasteurised producer-distributor bulk milk (PDBM). A total of 258 samples were collected from purchase points in 8 provinces in South Africa. The samples were tested for antibiotic residues, phosphatase, total aerobic bacteria, coliforms and *E. coli* counts. Matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) was used for identification of isolates. *E. coli* isolates were characterised for virulence factors, antimicrobial resistance, serotypes and presumptive *E. coli* O157:H7. Antibiotic residues and alkaline phosphatase were detected in 2% of both raw and pasteurised PDBM (n=258) and 21% pasteurised PDBM (n=104) samples respectively. A total of 729 isolates belonging to 21 genera and 59 species were identified. *E. coli*, *Enterobacter cloacae, Klebsiella oxytoca* and *Raoultella ornithinolytica* were the most abundant species. Spoilage Enterobacteriaceae species exceeded 50% of the total isolates. *E. coli* was detected and isolated from 36% of the milk samples. Thirty one *E. coli* isolates harbored virulence genes *stx1*/*stx2* and 38% (n=121) were presumptive O157:H7. Prevalence of samples with presumptive shigatoxin producing *E. coli* (STEC) was 10%. Antimicrobial resistant *E. coli* isolates were detected in 70% of the milk samples with 36% of *stx1*/*stx2* positive *E. coli* showing multi-drug resistance. Information obtained from the study will be used for modelling the public health risk posed by milkborne pathogens in PDBM, which in many cases is consumed by the poor and vulnerable population.

3.2. INTRODUCTION

Members of the Enterobacteriaceae family have been implicated in many safety and spoilage problems (Baylis et al., 2011). Enterobacteriaceae can enter an inadequately handled dairy chain

and cause enzymatic breakdown of proteins or lipids, instigating spoilage which contribute to substantial economic losses and wastage (Baylis et al., 2011). More so, some Enterobacteriaceae have emerged as potential opportunistic pathogens due to acquisition (via horizontal gene transfer (HGT) among different strains or bacterial species) of virulence and antibiotic resistance factors carried on mobile genetic elements such as plasmids, transposons and bacteriophage (Baylis et al., 2011). Enterobacteriaceae including; *E. coli* (STEC), *Salmonella* spp., and *Yersinia enterocolitica* have been detected frequently in milkborne disease outbreaks (Jayarao and Henning, 2001; Oliver et al., 2009).

Among members of Enterobacteriaceae, which are most implicated in serious fatalities and illnesses in most foodborne outbreaks, including milkborne disease outbreaks around the world, include several proficient *E. coli* pathotypes. Numerous outbreaks associated with Enteropathogenic *E. coli* (EPEC) and Enterohemorrhagic *E. coli* (EHEC) in milk and other foods have been reported recently (Oliver et al., 2009; EFSA-ECDC, 2012; EFSA, 2015). Pathogenic *E. coli* can cause disease in humans and animals due to an assortment of virulence factors (Beutin et al., 2004). One such *E. coli* pathotype which has been reported in milk outbreaks is the STEC which is a causative agent of haemorrhagic colitis and haemolytic uremic syndrome (HUS) (Oliver et al., 2009). The main route of infection with STEC in humans is via consumption of contaminated foods, including milk, which is commonly associated with asymptomatic dairy cattle reservoirs (Iweriebor et al., 2015). Important STEC virulence encoding genes, which make *E. coli* an insidious threat to food safety, are the phage-encoded shigatoxin genes (*stx* 1 and *stx* 2) and the intimin gene (*eae*). Shigatoxins inhibit protein synthesis of host cells and leads to cell death, while the intimin gene mediates colonisation. The enterohemolysin gene (*ehxA*) encodes for cell lyses whereby the membranes of target cells including erythrocytes, leukocytes and renal tubular cells

are perforated (Kerényi et al., 2005). The most implicated STEC in human illnesses associated with foods is the *E. coli* serotype O157:H7. However, current studies have revealed global emergence of non-O157 STEC serotypes (O111, O26, O145 and O103) associated with foodborne outbreaks (Constantiniu, 2002). Recent milkborne outbreaks associated with STEC O157:H7 have been reported in 2005, 2006 and 2007 in USA (CDC, 2008), 2008 in Germany and 2012 in Finland (EFSA, 2015). The presumptive route of O157 STEC in milk is through fecal contamination during milking and also direct shedding from infected udders (King, 2007).

The potential occurrence of pathogenic *E. coli* in food is not the only risk. In addition to the outbreaks, treatment of *E. coli* infections is now threatened by a rapid evolution of antimicrobial resistant strains (da Silva and Mendonça, 2012). Resistance of *E. coli* and other bacteria to antimicrobials has been reported worldwide and is one of the most pressing public health issues of our time. Inappropriate selection, intensive usage and abuse of antimicrobials may have led to antimicrobial resistance in bacteria (da Silva and Mendonça, 2012). Concerns have been raised about the potential spread of antibiotic resistance genes such as beta-lactams from food animal products such as milk to humans through the food supply (Iweriebor et al., 2015). Research has also revealed that there is an association between resistance to drugs and virulence capacity (da Silva and Mendonça, 2012). Acquisition of antibiotic resistance and virulence factor determinants in *E. coli* can be through lateral gene transfer in the environment or in the human and animal gut. The determinants can be found either inserted on the same plasmid or separately on bacterial chromosomal DNA and plasmids. These determinants might be co-selected by antibiotic selection pressure, hence, exacerbating the risk posed by *E. coli* to food safety (da Silva and Mendonça, 2012). Outbreaks of antibiotic resistance pathogenic bacteria have been reported recently in different foods (CDC, 2013b).

Occurrence of most milkborne disease outbreaks has been linked to consumption of raw milk at farms or milk which is sold directly from producer-distributor (PD) or from farm to consumer in bulk tanks (Jayarao et al., 2006; Oliver et al., 2009; EFSA-ECDC, 2012; EFSA, 2015). However, there are few reports on outbreaks from pasteurised milk (CDC, 2008). In developing countries, there are no available epidemiological statistics on milkborne outbreaks even though the sale of producer-distributor bulk milk (PDBM) is common. Suffice to say, in developing countries, the direct sale of milk to consumers plays an important role as it contributes to food security and nutrition as well as social and economic benefits (Swai and Schoonman, 2011). South Africa (SA) is one such country where the dairy industry is characterised by PDs selling milk directly from producers to consumers in form of bulk tank milk. The milk is mostly consumed by poor and vulnerable members of the population.

Recent reports have highlighted microbiological inadequacy in the quality of PDBM sold in South Africa. However, these studies have not characterised spoilage microbiota and possible pathogens in the milk. Therefore, this study focuses on characterisation of *E. coli* and other Enterobacteriaceae in PDBM. It is envisaged that the information from this research will be useful in modelling the risk from pathogenic *E. coli* in retail producer-distributor bulk milk and the proposal of potentially effective mitigation efforts towards enhanced food security through reduction in incidences of milkborne zoonosis.

3.3. MATERIALS AND METHODS

3.3.1. Milk sample collection

Sampling of PDBM was done from purchase points according to ISO standards (ISO, 1997) within 8 different geographical provinces (P1- P8) in SA by environmental health officers (EHOs). An

average of 30 retail bulk milk samples were collected per province adding up to a total of 258 samples (154 raw and 104 pasteurised). Each of the 500 ml milk sample was collected in sterile containers which were kept chilled and analysed at Lactolab Irene, Pretoria, SA, within 48 h of collection.

3.3.2. Antibiotic residue and phosphatase test

The alkaline phosphatase (ALP) test was carried out using PhosphatesmoMI test kit (MACHEREY-NAGEL GmbH & Co.KG, Germany). A CMT Antibiotic test kit (CHR HANSEN, Hoersholm, Denmark) (code number 611683) was used to detect the presence of antibiotic residues in the milk.

3.3.3. Microbiological analyses

3M™ petrifilm™ (Minisota, USA) were used for enumeration of total aerobic plate count, *E. coli* and coliforms. Total plate count was carried out on 3M™ Petrifilm™ Aerobic Count plates and 3M™ Petrifilm™ *E. coli* /coliform plates were used to detect and enumerate *E. coli* and coliforms (37 °C±0.5 °C for 24 h±2 h). Isolation and characterisation of *E. coli*, coliforms and other Enterobacteriaceae was carried out at the Department of Food Science, University of Pretoria. Five colonies from each *E. coli*/coliform petriflm plate were selected based on colour, size and morphology. Colonies were then streaked and purified on nutrient agar (Oxoid, UK) (18-24 h at 37 °C±0.5 °C). Eosin methylene blue and sorbitol MacConkey agar (Oxoid) were used for further presumptive confirmation of *E. coli* and coliforms (37 °C±0.5 °C for 24 h±2 h). Potassium hydroxide (3%) was used for Gram stain reactions (Romano et al., 2005).

3.3.4. Identification and characterisation of the bacterial isolates

A method by Mazzeo et al. (2006) and Pinto et al. (2010) was used for identification of the isolates. Bacterial cultures in nutrient agar were extracted and transferred directly to the MALDI-TOF steel-polished target plate, consisting of 8 wells per isolate, and overlaid with alpha-cyano-4 hydroxycinnamic acid matrix (Bruker, Germany) according to manufacturer instructions (Bruker Daltonik, Bremen, Germany). Mass spectra were generated with the Microflex LT mass spectrometer operated by the MALDI-Biotyper automation control and recorded by Flex Control software (Bruker Daltonics, Bremen, Germany). Three hundred shots per sample spot were acquired using the instrument settings consisting of linear positive mode, 60 Hz laser frequency, 20 kV acceleration voltage, 16.7 kV IS2 voltage, 170 ns extraction delay, and 2,000 to 20,137 m/z range as recommended for bacterial identification. The peak lists generated were used to directly obtain matches against the reference library (SARAMIS database) using the integrated pattern matching algorithm of the software (MALDI-TOF 3.0, Bruker Daltonics). MALDI Biotyper 3.0 software was used to analyse raw spectra of the bacterial isolates, with default settings. The software compares acquired sample spectra to reference spectra in the provided SARAMIS database and compiles a list of best matching database records. MALDI-TOF identification results were recorded as score values (SV) and used to determine the identity of the organism as proposed by the manufacturer. A score <1.700 indicates no identification, 1.700-1.999 indicate identification to genus level, ≥2.000 denotes identification to genus and probable species and scores above 2.3 indicate highly probable species identification (Cherkaoui et al., 2010).

3.3.5. Antimicrobial susceptibility testing of *E. coli* isolates

The Kirby-Bauer disk diffusion method (Sayah et al., 2005) was used to determine antibiotic sensitivity profiles of the isolated *E. coli*. A total of 11 antimicrobial agents (Oxytetracycline 30 µg, Cephalothin 30 µg, Nitrofurantoin 300 µg, Amoxicillin 25 µg, Ampicillin 10 µg, Neomycin 30 µg, Amikacin 30 µg, Gentamicin 10 µg, Nalidixic acid 30 µg, Chloramphenicol 30 µg, Norfloxacin 10 µg) (Mast Diagnostics, UK) were chosen on the basis of common usage for treatment in the medical and agricultural fields or as feed additives to promote growth in animals (Sayah et al., 2005). Isolates were categorised as susceptible or resistant to each antimicrobial agent (CLSI, 2011).

3.3.6. Serotyping

Serotyping of the *E. coli* isolates was performed by agglutination tests using polyvalent antisera (Antiserum *E. coli* TRIVAKENT III; BIO-RAD, Paris, France) against eleven common O antigens O111, O55, O26, O114, O124, O142, O125, O126, O128, O86 and O127.

3.3.7. Detection of virulence genes in *E. coli*

Multiplex real time PCR was used to detect virulence factors; *shigatoxin*1 (*stx 1*), shigatoxin2 (*stx 2*) and intimin (*eae*) in *E. coli* isolates. The thermocycler (C1000 Touch ThermalCycler CFX96TM Real Time System, Bio-Rad) and software (CFX 127 Manager IDE, Bio-Rad) were set up for analysis using iQ-Check™ STEC VirX catalogue # 128 357-8139 (Bio-Rad, Hercules, California) (Aijuka et al., 2014).

3.3.8. Presumptive detection of shigatoxin O157:H7 *E. coli*

Presumptive detection of O157:H7 *E. coli* was carried out using three selective media: BBL™ CHROMagar™ O157 agar (CHROMagar, Paris, France) with cefixime-tellurite (CT) selective supplement (Fluka analytical); BBL™ CHROMagar™ STEC with STEC ST162(S) supplement (CHROMagar Paris France); and Sorbitol MacConkey Agar (Merck, SA) supplemented with CT. *E. coli* isolates were incubated at 37 °C±0.5 °C for 24 h±2 h. *E. coli* O157:H7 (ATCC 35150) and *E. coli* ATCC (25922) was used as a positive and negative control respectively.

3.3.9. Statistical analysis

The data was analysed with STATA® version 11 (StataCorp, Texas, USA). The Chi-square test ($P < 0.05$) was used to test for significant differences in *E. coli*, coliforms and total aerobic counts between raw and pasteurised milk samples. Two-sided Fisher's exact test was used to test for correlation between virulence factor and antibiotic resistance capacity. Analyses of variance (one way) was performed to test for significant differences in antibiotic resistance patterns of *E. coli* isolated from PDBM in different provinces at 95% confidence interval. Raw spectra of the *E. coli, S. liquefaciens* and *K. oxytoca* strains were analysed using MALDI Biotyper 3.0 software in order to determine the association (clustering) of the isolates with source province/region. Cross-wise minimum spanning (MSP) tree matching was used to create a dendrogram. Similar MSPs result in a high matching score value. Each MSP is matched against all MSPs of the analysed set. The list of score values was used to calculate normalised distance values between the analysed species resulting in a matrix of matching scores. The visualisation of the respective relationship between the MSPs was displayed in a dendrogram using the standard settings of the MALDI Biotyper 3.0 software (Dubois et al., 2010). Distance values in a dendrogram were relative and normalised to a

maximal value of 10. Species with distance levels under 1.5 have been described as reliably classified into distinct strains (Sauer et al., 2008).

3.4. RESULTS AND DISCUSSION

3.4.1. Total plate count, coliforms and *E. coli* counts in retail PDBM

There was a significant difference between raw and pasteurised milk ($P< 0.05$) in mean log counts of total plate count, coliforms and *E. coli* (Figure. 3.1). Total aerobic plate count, coliforms and *E. coli* counts for raw milk ranges were 3.4 – 6.0, 2.4 – 2.7 and 2.0 – 2.1 log cfu/ml respectively (Figure. 3.1a). Prevalence of raw milk samples above SA national standards (SA, 2001 Act (54), (1972)) were 42%, 88% and 68% for *E. coli*, coliforms and total aerobic plate counts, respectively. The results on microbial counts from raw PDBM, where more than 60% of the samples were above the national standard, were consistent with previous studies carried out in SA (Lues et al., 2003; O'Ferrall-Berndt, 2003; Lues et al., 2010) and concur with studies in other countries e.g. Torkar and Teger (2008) in Slovenia, Pyz-Łukasik et al. (2015) in Poland and Van Kessel et al. (2004) in USA. These studies highlighted animal and equipment cleanliness, feeding and housing strategies, rinsing water for milking machine and milking equipment washing, mastitis in animals, milk transportation and storage conditions as reasons for high microbial counts in raw milk. High prevalence of coliforms and *E. coli* in raw milk is a cause of concern due to its association with contamination of fecal origin and the consequent risk of enteric pathogenic organisms in food. Total aerobic plate counts, coliforms and *E. coli* counts for pasteurised milk ranges were 2.2 – 4.8, 0 – 2.5 and 0 – 2 log cfu/ml respectively (Figure. 3.1b).

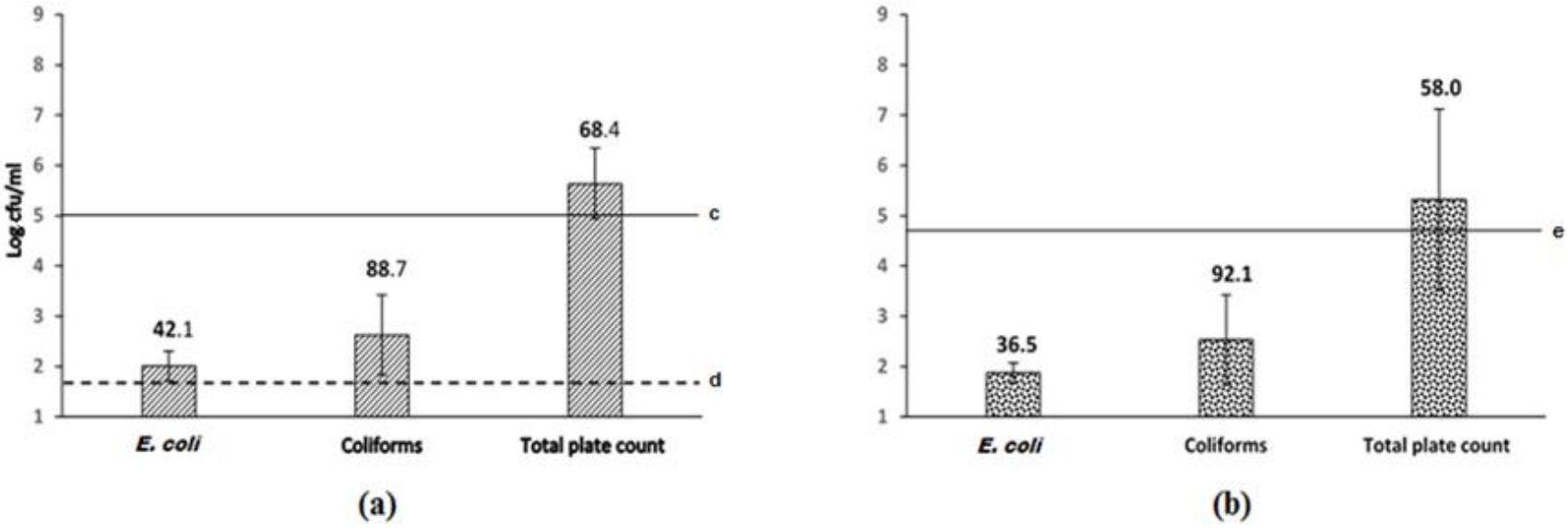

Figure 3.1: Mean *E. coli*, coliform and total aerobic plate count (log cfu/ml) for raw bulk milk (**a**) and pasteurised bulk milk (**b**) from producer-distributors in 8 provinces in South Africa.

*Solid and broken horizontal lines designated (c) and (d) on the graph, represent maximum limit for total plate count and coliforms in raw milk, respectively. Solid line designated (e) represent maximum limit for total plate count in pasteurised milk. Numbers above the graphs represent percentage of samples above Foodstuffs, Cosmetics and Disinfectants Act (54), (1972) limit for raw milk (3.1a) and pasteurised (3.1b). Coliforms should not be detected in pasteurised milk and *E coli* should not be detected in both raw and pasteurised milk. Error bars represent SD.

The presence of high aerobic plate counts in pasteurised milk can be attributed to cross-contamination, however, the issue of some thermoduric microorganisms which are reported to resist heat treatment in food cannot be ignored (Mercer et al., 2015).

There were 21% of pasteurised milk samples which were positive for ALP. The presence of ALP can be due to ineffective pasteurisation or poor milk handling after pasteurisation. Conversely, bacterial counts greater than 1.2×10^7 cfu/ml, as experienced in this study, can contribute sufficient microbial ALP that cause a false positive ALP test (Rankin et al., 2010). Again, ALP reactivation also results in a positive ALP test (Whitaker et al., 2003). Microbiological counts in 60% of the milk samples exceeded SA regulatory standards which suggests the need to assess the public health risk posed to consumers by retail PDBM in the country.

3.4.2. Identification and characterisation of bacterial species

A total of 729 Enterobacteriaceae and other bacterial isolates were detected and identified from 258 PDBM samples. The isolates belonged to 21 genera and 59 species. Prevalence of the dominant Enterobacteriaceae species isolated from raw and pasteurised PDBM (coded 1-59) is depicted in Table 3.1 and their distribution in raw and pasteurised PDBM in the respective provinces is presented in Figure 3.2. Raw and pasteurised retail bulk milk samples from all the 8 provinces were contaminated with a wide diversity of Enterobacteriaceae species. More than 50% of the Enterobacteriaceae isolated and detected in the current study were spoilage psychrotrophic microbiota. *Pseudomonas* spp., was the dominant psychrotrophic bacteria in all the provinces. A commercially important characteristic of psychrotrophs that were isolated in this study, is their ability to grow at low temperatures (3–7 ^{o}C) and release of enzymes that hydrolyse and use large protein molecules and lipids for growth ultimately causing milk spoilage (Ledenbach and

Marshall, 2010). The released enzymes are heat stable and are not inactivated by pasteurisation or by other heat treatments and may continue to degrade milk and its products, even when the bacterium is destroyed (Ledenbach and Marshall, 2010).

The most prevalent Enterobacteriaceae species from PDBM, in decreasing order, were *E. coli*, *R. ornithinolytica, K. oxytoca, E. cloacae, E. asburiae, Serratia liquefaciens* and *Hafnia alvei*. These bacteria were also the spoilage microbiota isolated in high numbers in food products as by Doulgeraki et al. (2011). The Enterobacteriaceae are also considered as opportunistic pathogens although their implication in diarrheal diseases is not clear with the exception for *E. coli* pathotypes. More so, Enterobacteriaceae species in other studies have been noted to harbor antibiotic resistance and multi-drug resistance capacities (Miranda et al., 2008). Coque et al. (2008) reported Enterobacteriaceae as reservoirs of extended-spectrum β-lactamases (ESBLs) in bulk milk environments. *R. ornithinolytica* were the most prevalent species in pasteurised PDBM samples (Table 3.1). Most reports of *R. ornithinolytica* (formerly *Klebsiella ornithinolytica)* were associated with incidences of food poisoning (Lin et al., 2012). *R. ornithinolytica* has histamine-producing capabilities, due to *hdc* genes, which encode histidine decarboxylase (Lin et al., 2012). There are only a few studies on this bacteria in milk and its products and the real importance of *R. ornithinolytica* as a food spoilage organism or an enteric pathogen is underestimated (Lin et al., 2012). Detection of *S. liquefaciens, K. oxytoca and H. alvei* in this study is of commercial significance in milk. Their presence in milk has not only been linked to undesirable alteration in appearance and flavours but also to residual activity of enzymes such as proteases which have been associated with gelation of milk and coagulation of milk proteins during storage (Nornberg et al., 2010).

Table 3.1: Prevalence of dominant Enterobacteriaceae and other bacterial species isolated from raw and pasteurised producer-distributor bulk milk in South Africa (n=258).[1]

Species code	Species name	RM (%)	PM (%)	Total number of isolates
3	*Acinetobacter guillouiae*	0.6	0.5	4
13	*Buttiauxella agrestis*	-	2.3	5
14	*Buttiauxella. gaviniae*	0.4	1.9	6
15	*Buttiauxella noackiae*	-	3.2	7
17	*Citrobacter braakii*	3.3	3.2	23
18	*Citrobacter freundii*	2.1	2.3	15
21	***Escherichia coli***	**22.8**	**5.6**	**121**
23	*Enterobacter amnigenus*	3.1	4.2	24
24	***Enterobacter asburiae***	**7.5**	**3.2**	**43**
25	***Enterobacter cloacae***	**7.5**	**7.4**	**52**
26	*Enterobacter kobei*	0.8	1.4	7
27	*Enterobacter ludwigii*	2.5	1.4	15
28	*Ewingella Americana*	0.4	1.4	5
29	***Hafnia alvei***	**4.4**	**14**	**52**
30	***Klebsiella oxytoca***	**10.2**	**9.7**	**70**
31	*Klebsiella pneumoniae*	2.9	0.9	16
33	*Kluyvera intermedia*	1	0.9	7
35	*Pantoea agglomerans*	1.5	0.9	9
36	*Proteus mirabilis*	0.4	-	2
37	*Proteus vulgaris*	0.2	0.5	2
39	*Pseudomonas aeruginosa*	0.2	0.5	2
43	*Pseudomonas fluorescens*	0.2	1.4	4
47	*Pseudomonas lundensis*	0.4	-	2
48	*Pseudomonas nitroreducens*	-	0.9	2
52	*Rahnella aquatilis*	0.8	2.3	9
53	***Raoultella ornithinolytica***	**16.1**	**17.1**	**114**
54	*Raoultella terrigena*	0.4	0.5	3
55	*Serratia fonticolla*	0.4	-	2
56	***Serratia liquefaciens***	**6.3**	**8.3**	**48**
57	*Serratia marcescens*	1.5	1.9	11
58	*Stenotrophomonas maltophilia*	0.2	0.5	2
59	*Yersinia enterocolitica*	1.7	1.9	12
	Total number of isolates	479	216	697

*[1]RM – Raw milk. PM – Pasteurised milk. (-) not present. Figures in bold represent the most abundantly isolated species.

Six different species of the genus *Enterobacter* were identified from PDBM, however, *E. cloacae* and *E. asburiae* were detected at higher prevalence. *E. cloacae* and *E. asburiae* are emerging opportunistic pathogens causing nosocomial infections and are frequently detected in infant formula milk (Boerlin et al., 2001).

More diverse species were isolated in pasteurised milk than raw milk in this study. Out of the recorded 59 species, 19 were isolated from pasteurised milk only while 10 were obtained from raw milk only, and a total of 30 species were common to both (Figure 3.2).

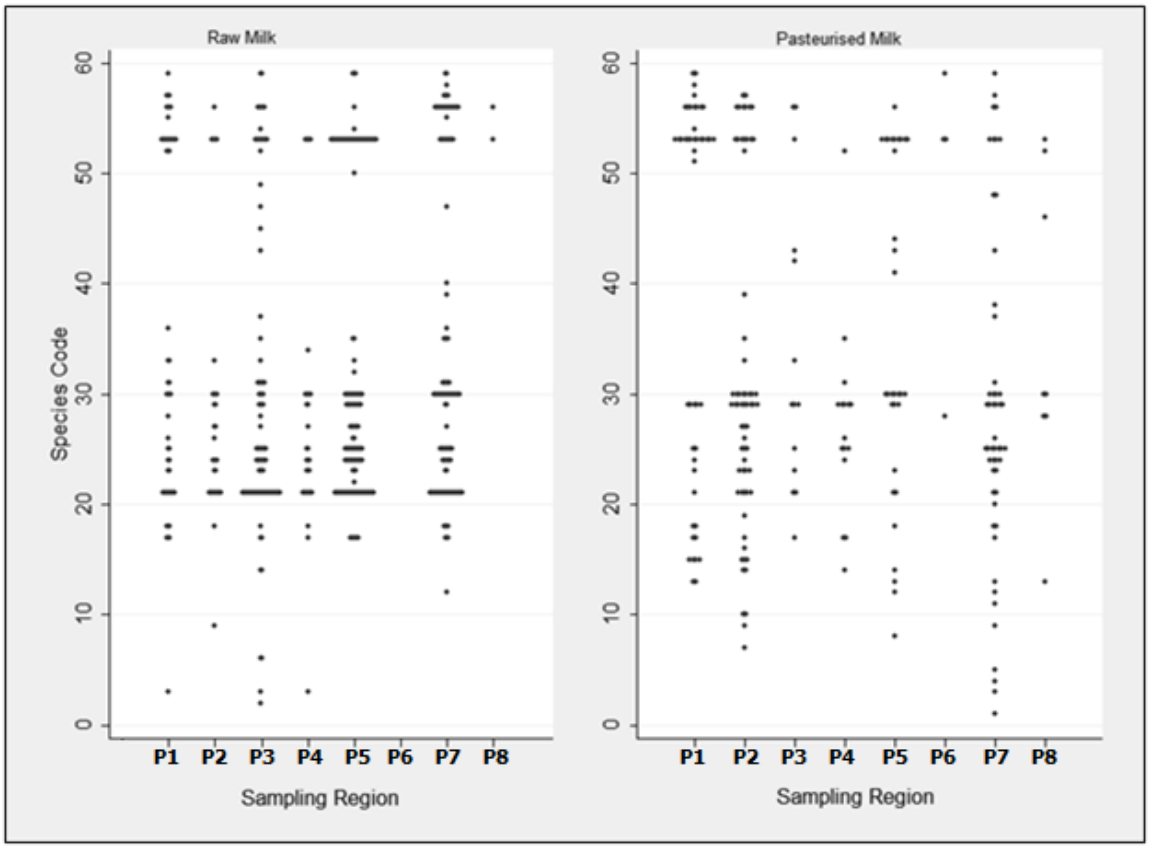

Figure 3.2: Distribution of Enterobacteriaceae and other bacterial species (n=729) from raw and pasteurised producer- distributor bulk milk from 8 different provinces in South Africa (P1 – P8).

*Isolated species were coded 1 to 59. Width of the band represents the abundance of a particular species in the province. Species names for the dominant Enterobacteriaceae species are given in Table 3.1.

The species of the organisms that were exclusively present in pasteurised milk (belonged to the following genera; *Acinetobacter, Aeromonas, Buttiauxella, Citrobacter, Pseudomonas, Raoultella* and *Serratia*) are predominantly waterborne microorganisms. This suggests a potential cross contamination from the processing environment arising from water aerosols and improperly cleaned processing and handling equipment (Eneroth et al., 2000). In addition, the post-process contamination can arise from multi-species biofilms established in processing equipment forming major reservoirs for contamination (Chmielewski and Frank, 2003). *S. liquefaciens* and *K. oxytoca* were found in milk samples from 8 and 7 provinces, respectively, while 12% of the species were isolated from at least 78% of the samples (Figure. 3.3).

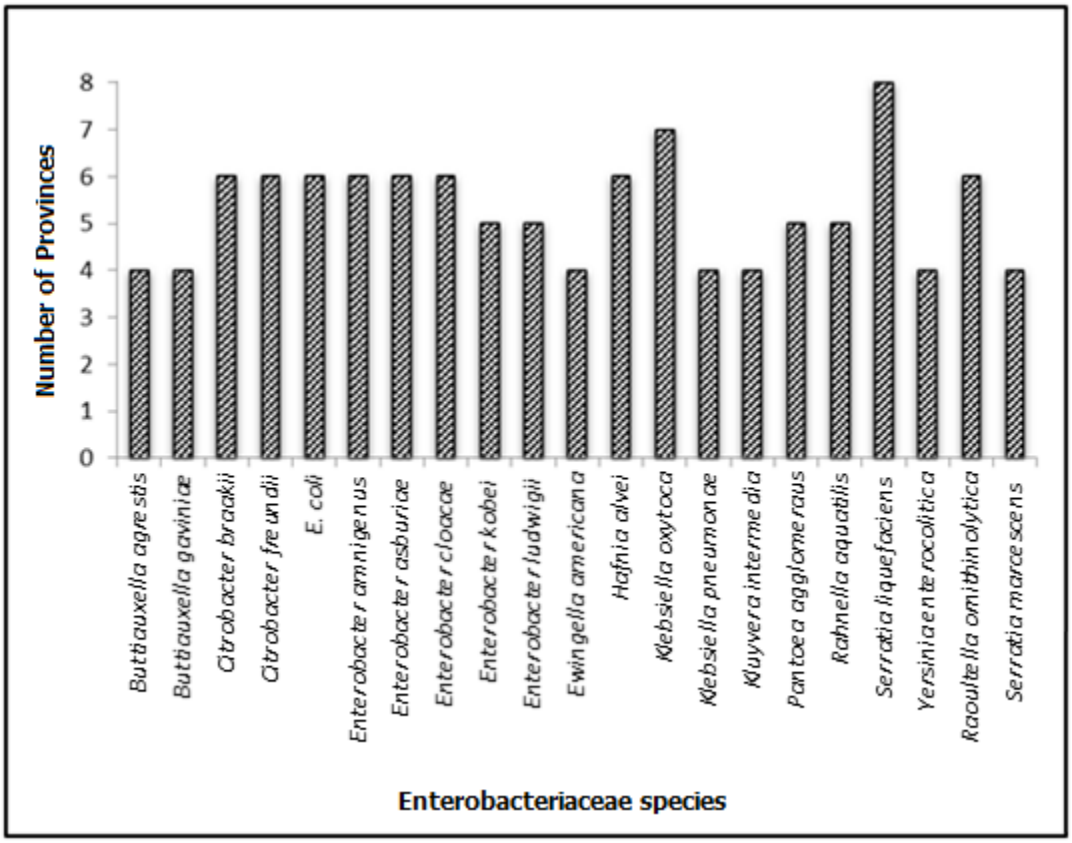

Figure 3.3: Frequency of dominant Enterobacteriaceae and other bacterial species isolated from raw and pasteurised producer-distributor bulk milk from 8 provinces in South Africa.

Yersinia enterocolitica was also identified in 12 out of 258 PD milk samples. Two out of 12 isolates were isolated from 2 pasteurised PDBM samples. *Y. enterocolitica* has been reported as an important public health hazard (Jayarao and Henning, 2001; Jayarao et al., 2006; Oliver et al., 2009) and has been frequently isolated in milkborne outbreaks in the USA and Europe (Oliver et al., 2009; EFSA, 2015). In the USA, Jayarao and Henning (2001) carried out a survey and reported 8 pathogenic *Y. enterocolitica* strains isolated from 131 bulk tank milk samples. The virulence capacity of *Y. enterocolitica* strains identified in the present study was, however, not tested. Important to note is that *Y. enterocolitica* can also resist heat treatment and is able to grow to large numbers below 15 ^{o}C (refrigeration temperatures) which makes contaminated milk a significant health risk (Jayarao and Henning, 2001).

3.4.3. Characterisation of *E. coli*

E. coli was isolated from 36% (n=258) of all the samples, with more raw milk samples (59%) testing positive for *E. coli* than pasteurised milk (40%) (Figure. 3.4). Only milk samples from two provinces were negative for *E. coli*. All 121 *E. coli* isolates tested negative for the 11 tested serotypes. Van Kessel et al. (2004) reported that presence of *E. coli* in raw milk can be via intramammary secretion or via fecal contamination of the udder or milking equipment. In the present study, 2% (n=258) PDBM samples tested positive for antibiotic residues. The risk of exposure to antimicrobial residues in milk has previously been reported (Kurwijila et al., 2006).

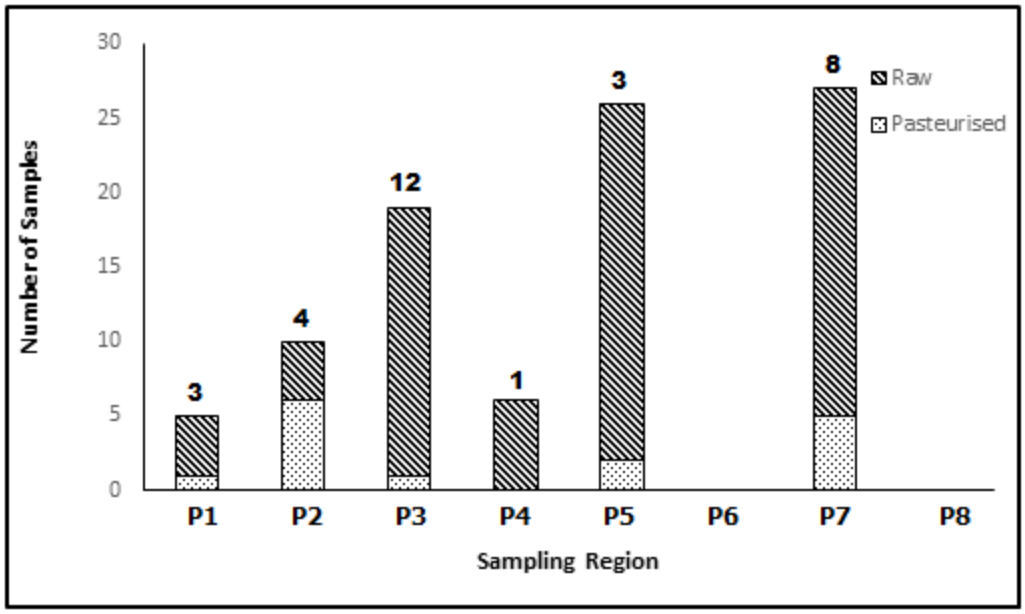

Figure 3.4: Number of samples which tested positive for *E. coli* from raw and pasteurised producer-distributor bulk milk from the 8 provinces in South Africa.

*Figures above the graphs represent number of *E. coli* isolates which tested positive for either *stx* 1/*stx* 2.

Studies have reported a significant association between veterinary antibiotic residue in animal products and obesity in humans (Riley et al., 2007). However, there are disagreements when it comes to the association between antimicrobial residue in food and subsequent development of antimicrobial resistant bacteria. Antimicrobial resistant *E. coli* isolates were detected in more than 70% of the milk samples. Out of the 121 isolates tested, 73% showed resistance to at least one of the 11 antibiotics (Table 3.2). Resistance to antibiotics in *E. coli* from provinces P3 was significantly higher ($p < 0.05$) than in all the other five provinces. A higher number of isolates was resistant to cephalothin and susceptible to nalidixic acid. Studies around the world on *E. coli* from different sources have also identified high resistance to the antimicrobial agent cephalothin (Erskine et al., 2002; Schroeder et al., 2002; Sayah et al., 2005). Cephalothin is a cephalosporin which is not used in food animals as a feed additive or as medicine (Sayah et al., 2005). However,

high resistance to cephalothin may be attributed to cross-resistance which develops through using other cephalosporins (Prescott et al., 2000; Sayah et al., 2005).

Table 3.2: Prevalence of antibiotic resistant *E. coli* isolated from producer-distributor bulk milk in South Africa.

Antibiotic	**Provinces**						
	P1 (n=10)	**P2** (n=15)	**P3** (n=30)	**P4** (n=7)	**P5** (n=32)	**P7** (n=27)	**Resistance (n =121)**
Oxytetracycline	10	33.3	23.3	12.5	15.6	7.6	**17.4**
Cephalothin	50	40	70	37.5	53.1	38.4	**51.2**
Nitroforantoin	0	6.7	10	0	3.1	15.3	**7.4**
Amoxicillin	40	6.7	36.7	12.5	21.9	15.3	**23.1**
Gentamicin	0	6.7	20	0	9.4	0	**8.3**
Neomycin	20	20	56.7	50	37.5	15.3	**34.7**
Amikacin	0	0	16.7	0	0	7.6	**5.8**
Norfloxacin	0	6.7	6.7	0	0	0	**2.5**
Nalidixic acid	0	0	6.7	0	0	0	**1.7**
Chloramphenicol	0	0	10	0	0	3.8	**3.3**
Ampicillin	20	6.7	46.7	12.5	25	15.3	**24.8**

*Values in Table 3.2 represent % of resistant isolates. n- is number of *E. coli* isolates per province.

Increased susceptibility of drugs such as nalidixic acid and norfloxaxin that was noted in the *E. coli* isolates can be as a result of restricted use of these agents in livestock production. Hence, the resistant determinants are lost over time without exposure to the drugs (Prescott et al., 2000). Approximately 60% (n=89) of the antibiotic resistant isolates were confirmed multidrug resistance (MDR). The most prevalent MDR patterns were resistant to 2 and 3 classes of antibiotic agents, however, more than 3% of isolates in provinces P2 and P3 exhibited MDR resistance to more than

three different classes of antibiotic agents. The highest number of phenotypes was observed for single antibiotic cephalothin (KF) (15.7%) followed by oxytetracycline (OT) (5%). Province P5 had the highest phenotypes which were resistant to KF. Cephalothin was found in all MDR phenotypes observed. Provinces P3, P5 and P7 had the highest diversity of the MDR phenotypes for the antibiotics. *E. coli* may develop multi drug resistance either independently and simultaneously, or co-selection of resistance factors by the organism (Sayah et al., 2005). High resistance of the *E. coli* isolates to cephalothin (51% isolates), neomycin (34% isolates), ampicillin (24% isolates), amoxicillin (23% isolates) and oxytetracycline (17% isolates) suggest the exposure of the isolates to the antibiotics. These antibiotics belong to commonly used classes of antibiotics including cephalosporins, tetracyclines, aminoglycosides and penicillins in SA for treatment, prophylaxis and as growth promoters in food-producing animals (Moyane et al., 2013). Kim et al. (2007) suggested use of antibiotics in treatment of cattle mastitis as a factor influencing increased prevalence of antibiotic-resistant pathogens though there is a lack of consensus concerning the link between drug usage and subsequent resistance in bacteria. Human food safety concerns has been raised in cases where drug resistance bacteria is isolated in food since there is a potential transfer of resistant foodborne pathogens to humans thought out the food chain (Moyane et al., 2013). The highest resistance from the *E. coli* isolates in this study was noted in β-lactam antibiotics. β-lactam antibiotics has low toxicity, a factor that has resulted in overuse of these drugs within animal husbandry and the medical fraternity (Moyane et al., 2013). A few studies have noted Enterobacteriaceae resistance to β-lactam antibiotic in milk samples, however, a study by Geser et al. (2012) reported resistance in *E. coli* from milk samples to CTX-M β-lactam antibiotics. Resistance to β-lactam antibiotics and the aminoglycosides was detected in *E. coli* isolates (73%,

n=121) in PDBM sampled from all the provinces. This may be as a result of the *E. coli* from different provinces in SA being exposed to similar antibiotics despite the geographical difference.

There was a higher prevalence in multiple antibiotic resistant *E. coli* from provinces P2 and P3 than that of other provinces. Multiple resistance to antibiotics by bacteria may be linked to acquired resistance either by cross-resistance or co-selection of antibiotic resistance determinants (Baker-Austin et al., 2006). In cases of cross-resistance in bacteria, a single resistance mechanism may confer resistance to an entire class or different classes of antibiotics and co-selection means that, a bacteria can acquire additional resistance through mobile genetic elements such as plasmids in a large pool of antibiotic resistance bacteria in the environment, ultimately resisting more than one antibiotic (Baker-Austin et al., 2006). For this reason, PDBM in the current study may serve as a reservoir of antibiotic-resistant pathogens and can pose a food safety risk to the public as new antibiotic resistant pathogenic Enterobacteriaceae strains can emerge through gene transfer.

Thirty one *E. coli* isolates were positive for virulence genes *stx1*/*stx2* and 38% (n=121) were presumptive O157:H7. A total of 16%, 13% and 12% (n=121) of the *E. coli* isolates were presumptively positive for O157:H7 on CT-SMAC, CHROMagar^{TM} O157 and BBL^{TM} CHROMagar^{TM} STEC agar respectively. Out of the 46 isolates which were presumptive positive for O157:H7 *E. coli*, 10% showed a positive result on at least two of the media. The intimin (*eae*) gene was absent in all the *E. coli* isolates (Table 3.3).

A proportion of 28% *stx1*/*stx2* positive *E. coli* were isolated in pasteurised PDBM samples. Six presumptive O157:H7 *E. coli* isolates also harbored *stx1*/*stx2* genes. Presence of virulence factors linked with enterohemorrhagic *E. coli* (EHEC) in PDBM suggested the ability to cause shigatoxin-related human infection and disease. Milkborne outbreaks and hospitalisation owing to this *E. coli*

pathotypes has been well documented and reported around the world (CDC, 2005; Oliver et al., 2009; EFSA-ECDC, 2012).

Table 3.3: Occurrence of presumptive O157:H7 strains and virulence factors (*stx* $_1$, *stx* $_2$) in *E. coli* isolates from raw and pasteurised producer-distributor bulk milk in South Africa.

Provinces	**O157:H7 %**	***stx*$_1$/*stx*$_2$ %**	**Total number of *E. coli* isolates**
P1	40.0	30.0	10
P2	53.3	26.7	15
P3	50.0	40.0	30
P4	14.2	14.2	7
P5	12.5	9.2	32
P7	51.8	29.6	27
Total number of isolates	**46** (38.0%)	**31** (25.6)%	**121**

Shigatoxin producing *E. coli* was present in 7% and 3%, n=258 of the raw and pasteurised PDBM samples, respectively. Several studies around the world have reported the presence of STEC only in raw milk samples in Italy (5.7%, n=123), USA (2.4%, n=228) (Jayarao and Henning (2001), and France (21%, n=205) (Perelle et al., 2007). The main route by which STEC enters the raw milk supply chain is through fecal contamination or intramammary secretions from infected cow udder (King, 2007).

The sensitivity and specificity of the media used for the identification of presumptive *E. coli* O157:H7 was not evaluated, though CT-SMAC showed the highest number of O157:H7 characteristic colony appearance. Manafi and Kremsmaier (2001) evaluated sensitivity and specificity of four different O157:H7 media SMAC, RainbowAgar O157:H7, Biosynth Culture

Medium O157:H7 and Fluorocult HC and found Biosynth Culture Medium to be the most sensitive medium. However, they strongly recommended a confirmatory test either by immunological or PCR methods for suspect EHEC colonies, irrespective of whatever combination of media used.

Identification of *stx* genes in presumptive O157:H7 from PDBM in SA demonstrates that this milk may present a risk to public health. *E. coli* O157:H7 is of great significance to foodborne diseases, because of its high virulence (it can cause disease at a dose of 5–50 cells) (Farrokh et al., 2013). Prevalence of STEC O157:H7 in bulk milk samples in this study (2%, n=258) was in the range of other studies, who worked on similar milk, carried out in the USA, (0.02%, n=859) (Karns et al., 2007), (2.4% n=248) (Jayarao et al., 2006), Greece (0.7%, n = 950) (Solomakos et al., 2009) and Australian milk (varied from 1 - 3%) (DairyAustralia, 2006). All presumptive O157:H7 *E. coli* from PDBM which harboured *sxt 1* and/or *stx 2* lacked the *eae* gene. This is contrary to other studies which noted an association between *stx* and *eae* genes in *E. coli* (Omisakin et al., 2003). The EHEC O157:H7 strain contains a locus of enterocyte effacement which is characterised by presence of either *eae, stx 1, stx 2* genes or a combination of all the three (Karns et al., 2007). Pathogenic *E. coli* lacking the *eae* gene has been reported to use other adhesion factors for attachment and pathogenicity (Doulgeraki et al., 2011). In similar findings to this study, STEC isolates from raw milk (44 isolates) did not harbor the *eae* gene (Farzan et al. (2012). The 80% (n=31) of *stx* positive *E. coli,* which were not O157:H7 suggested that the PDBM was also contaminated with emerging non-O157:H7 EHEC strains. Non-O157 *E. coli* pathotypes have been well documented in foodborne outbreaks which include milk and its products around the world (Constantiniu, 2002). Karns et al. (2007) reported a higher prevalence in non-O157 EHEC than O157:H7 and other serotypes in bulk tank milk. *E. coli* strains belonging to other serogroups (diarrheagenic) of public health concern may therefore pose health risk in PDBM in SA.

In both raw and pasteurised PDBM, 58% of *stx1*/*stx2* positive *E. coli* were resistant to at least one antibiotic which was tested. Multi-drug resistance (MDR) to at least two classes of antibiotics was recorded in 38% (n=31) of the *stx1*/*stx2* positive isolates. More than 50% of the *stx1*/*stx2* positive isolates were resistant to cephalothin, a beta-lactam first generation drug. Results of the two-sided Fisher's test revealed that there was no significant correlation between antibiotic resistance and presence of *stx1*/*stx2* genes. The dissemination of resistance is associated with genetic mobile elements such as pathogenicity islands that may also carry virulence determinants (da Silva and Mendonça, 2012). Apart from virulence traits and antibiotic resistance genes being on the same genetic island such as plasmids, *E. coli* can also acquire virulence factor-encoding prophase (on chromosomal DNA) or plasmid bearing antibiotic resistance factors (Rasko et al., 2011).

STEC (2 isolates) resistant to quinolones, were also isolated from PDBM in the current study. Quinolone resistance are reported to have an inverse relationship with virulence genes. Resistance to the quinolones is mediated by point mutation in DNA gyrases and topoisomerases (da Silva and Mendonça, 2012). Most researchers have argued the use of antibiotics such as quinolone in treatment of STEC infected patients because they have highlighted their administration to increase the risk of HUS (Muniesa et al., 2012). In this current study, prevalence of potential pathogenic EHEC which has drug resistance capacity, and also possibility of the organisms to grow under improper storage conditions can present a significant public health risk, especially to the vulnerable members of the community.

3.4.4. Hierarchical cluster analysis

Five main clusters (A to E) were observed from the *E. coli* dendrogram (Figure 3.5). The *E. coli* isolates were further subdivided into 8 sub-clusters defined by greater than 75% similarity which

was equivalent to a 1.5 distance level considered as demarcating similar strains. Isolates within the five main clusters grouped together irrespective of the province they were isolated. Group A comprised of isolates which emanated from five provinces, however, isolates from province P5 (40%, n=22) were dominant. Suffice to note, all the five main clusters (A – E) observed contained at least one isolate from the 6 different provinces. The 5 main cluster groups contained at least 4 *E. coli* isolates which had been found to carry *stx1*/*stx2* genes and came from different geographical locations. Isolates from P5 were found in all the sub-clusters. However, isolates from provinces P3 and P7 formed at least 80% of isolates from sub-clusters C5 and C8. Sub-cluster C4 was the biggest group consisting of 21% (n=121) of the *E. coli* isolates which were representative isolates from all the 6 provinces. This group consisted of isolates which previously had common resistance to cephalothin, neomycin, ampicillin, amoxicillin and oxytetracycline.

There was no particular trend with respect to origin of the isolates. The isolates grouped irrespective of the origin indicating that isolates from different provinces were related. Some of the isolates from certain provinces were found in all the cluster groups which indicates high genetic diversity among the isolates and wide distribution despite spatial differences in the milk sources. High genetic diversity of *E. coli* has been noted in isolates from human, animal, food and the environment (Aslam et al., 2003; Apun et al., 2006; Van Elsas et al., 2011). Reasons for high genetic diversity in the *E. coli* could be attributed to its versatility and high degree of genomic plasticity, via gene loss or gain and through lateral gene transfer (Rasko et al., 2008). Internationally standardised phage typing, subtyping of *stx* and *eae* genes, molecular fingerprinting using PFGE or REP-PCR have been used in studying epidemiological relationships and diversity among *E. coli* pathotypes (Beutin et al., 2002).

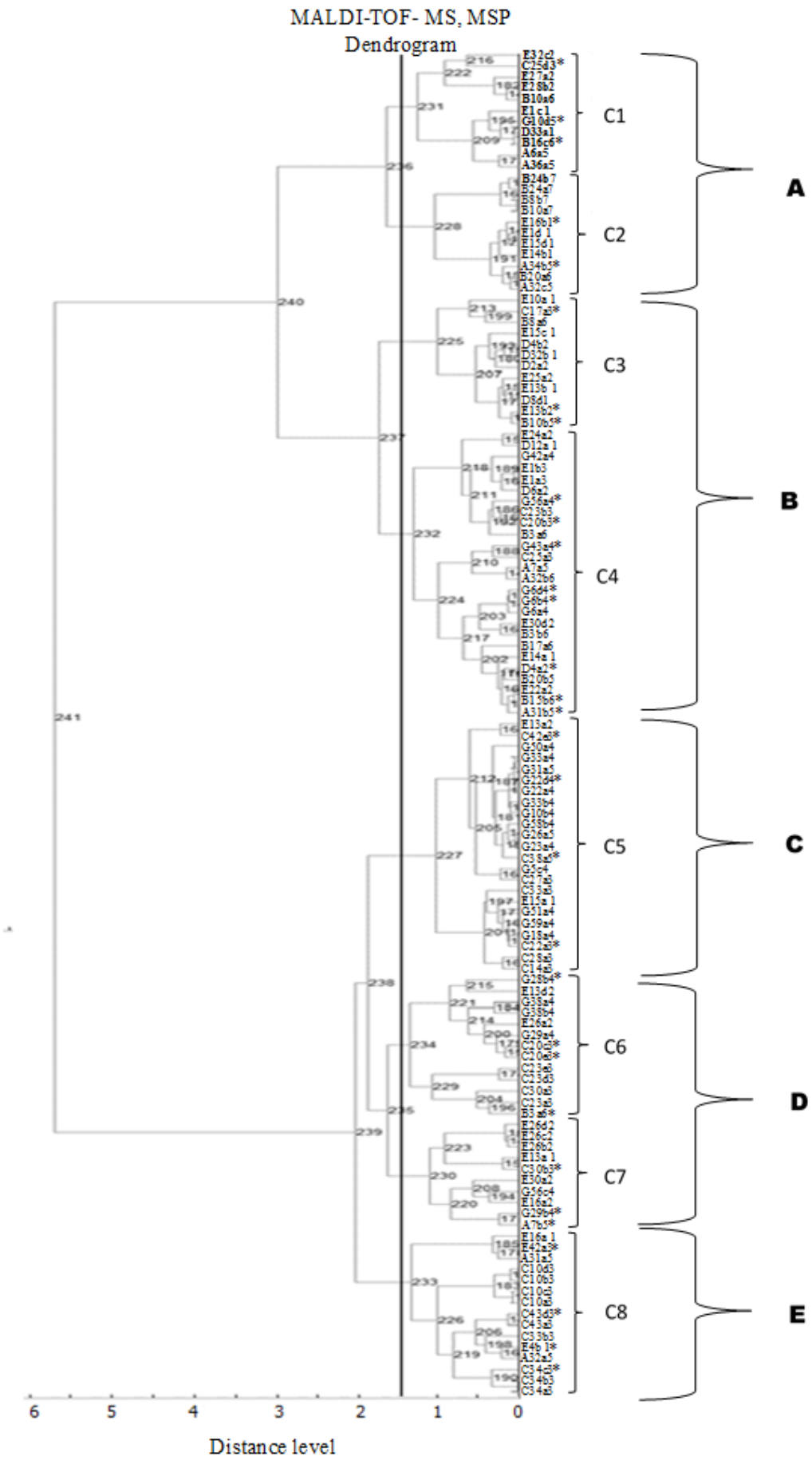
MALDI-TOF- MS, MSP
Dendrogram
C1
C2
C3
C4
C5
C6
C7
C8
A
B
C
D
E
6
5
4
3
2
1
0
Distance level

Figure 3.5: Two-dimensional hierarchical cluster analysis dendrogram for 121 *E. coli* isolates from producer-distributor bulk milk in South Africa.

*Capital letters represent the different geographical provinces while numbers with small letters represent different strains. The vertical line at 1.5 distance level represent the minimum similarity considered to be the highest point which distinct strains were identified (>75% similarity). Cluster groups under distance level 1.5 were considered to be distinct strains. Major brackets enclose the five super clustering groups (A to E) while the minor brackets enclose the sub-clusters (C1 to C8). Provinces were labelled as follows: *P1*- (A), *P2*- (B), *P3*- (C), *P4*- (D), *P5*- (E), *P6*- (F), *P7*- (G), *P8*- (H) Isolates with the coloured asterisk (*) were positive for *stx1/stx2*.

However, recent developments in mass spectrometry (MS) show that its accuracy in identification compares well (>80%) to conventional tools such as molecular techniques and API systems and have made it possible to use whole-cell MALDI-TOF MS in elucidating relationship among bacteria isolates from food and the environment (Risch et al., 2010; Arinto-Garcia et al., 2015).

Albeit, originating from different provinces, STEC from PDBM in the current study revealed genetic relatedness by grouping in unique classes. Clusters with *E. coli* isolates that had common antibiotic resistance patterns, even though they came from different regions, revealed possibility of common antibiotics use in different provinces in SA (da Silva and Mendonça, 2012).

S. liquefaciens and *K. oxytoca* were clustered because of their high occurrence and frequency of isolation in all the provinces (Figure 3.3) and also their significance in food safety as opportunistic pathogenic and food spoilage organisms. *S. liquefaciens* strains showed three main distinct clusters (A, B and C) and 6 sub-clusters (C1 to C6) from the 8 provinces. *S. liquefaciens* isolates were distributed irrespective of origin in all the three main clusters (Figure 3.6). Isolates from P1 were found in sub-clusters C2, C3, C4 and C6 and they also accounted for 70% of the C3 and C6 population. Sub-cluster group C1 was the biggest group which consisted of 25% (n=47) of the *S. liquefaciens* isolates and the majority of the isolates in C1 came from province P5.

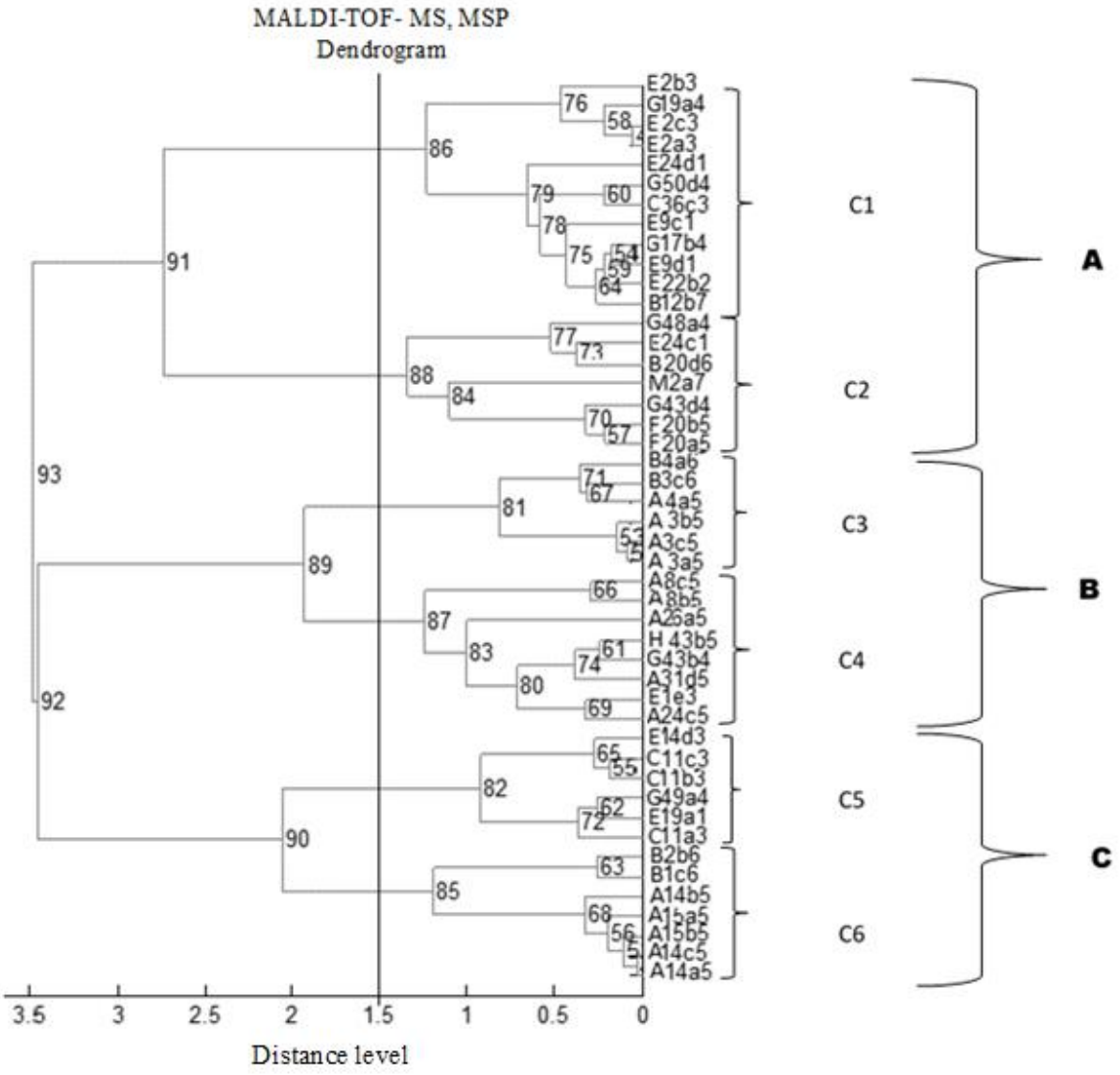

Figure 3.6: Two-dimensional hierarchical cluster analysis dendrogram for 47 *S. liquefaciens* isolates from producer-distributor bulk milk in South Africa.

*Capital letters represent the different geographical provinces while numbers with small letters represent different strains. The vertical line at 1.5 distance level represent the minimum similarity considered to be the highest point which distinct strains were identified (>75% similarity). Cluster groups under distance level 1.5 were considered to be distinct strains. Major brackets enclose the three super clustering groups (A, B, C) while the minor brackets enclose the sub-clusters (C1 to C6). Provinces were labelled as follows: *P1*- (A), *P2*- (B), *P3*- (C), *P4*- (D), *P5*- (E), *P6*- (F), *P7*- (G), *P8*- (H).

Sub-clusters from the *S. liquefaciens* dendrogram consisted of isolates from provinces which are in close geographically proximity.

A two dimensional hierarchical cluster analysis for *K. oxytoca* showed 8 sub-clusters from 3 distinct main clusters (A, B, C). Isolates within the main cluster groups A and B were grouped irrespective of the geographical source. Cluster group B contained 4 sub-clusters with isolates mostly from province P1 (Figure 3.7). Isolates from P7 formed 90% of the members of cluster group C. Sub-cluster C8 was the biggest consisting of 21% of the *K. oxytoca* isolates and 86% (n=15) were from province P7. Isolates from province P5 where found in all the sub-clusters. Isolates originating from provinces in close proximity, grouped together into sub-clusters.

S. liquefaciens and *K. oxytoca* isolates also showed no particular trend with respect to origin of the isolates. The isolates, like in *E. coli* cluster analysis, grouped irrespective of the origin indicating high genetic diversity as well. Due to genomic plasticity of Enterobacteriaceae through lateral gene transfer, plasmid-encoding genetic determinants give rise to genetic and ecological diversity in this family of bacteria (Brisse and Verhoef, 2001; Iguchi et al., 2014). Iguchi et al. (2014) characterised the genetic diversity of *Serratia* spp., using complete genome sequences and found a remarkable intra-species genetic diversity, both at the sequence level and with regards to genome flexibility, which may reflect the diversity in members of this species. Brisse and Verhoef (2001) noted a high degree of genetic heterogeneity and phylogenetic diversity of *K. oxytoca* from 120 clinical isolates using randomly amplified polymorphic DNA (RAPD) analysis. The authors noted that molecular techniques such as RAPD and 16S rRNA PCR have limitation in deducing strain relationship of Enterobacteriaceae species.

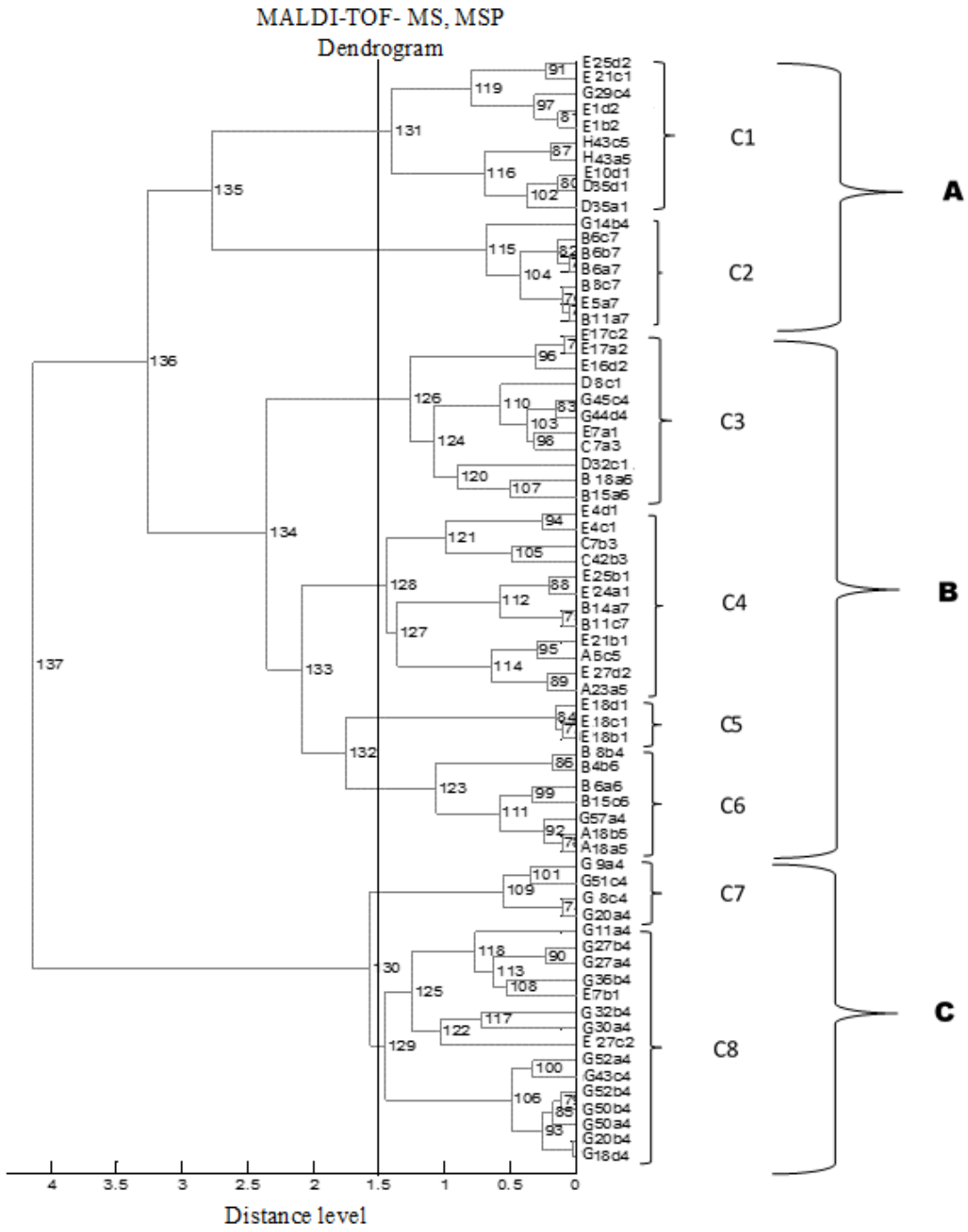

Figure 3.7: Two-dimensional hierarchical cluster analysis dendrogram for 70 *K. oxytoca* isolates from producer-distributor bulk milk in South Africa.

*Capital letters represent the different geographical provinces while numbers with small letters represent different strains. The vertical line at 1.5 distance level represent the minimum similarity considered to be the highest point which distinct strains were identified (>75% similarity). Cluster groups under distance level 1.5 were considered to be distinct strains. Major brackets enclose the three super clustering groups (A, B, C) while the minor brackets enclose the sub-clusters (C1 to C8). Provinces were labelled as follows: *P1*- (A), *P2*- (B), *P3*- (C), *P4*- (D), *P5*- (E), *P6*- (F), *P7*- (G), *P8*- (H).

Such shortcomings can be overcome by use of the MALDI-TOF-MS. The MALDI-TOF-MS techniques compares well with minimal limitations in bacteria characterisation (Maja et al., 2013), source tracking (Siegrist et al., 2007) and strain relationship (Pignone et al., 2006) between isolates from food and the environment. Based on this study, the analysis of level of diversity among strains originating from different locations using MALDI-TOF-MS can be helpful in typing possible clonal relationship among Enterobacteriaceae strains and to monitor the pathway of transmission of pathogenic strains that may pose a threat to public health and also the spoilage microbiota in food.

3.5. CONCLUSION

This study reports poor microbiological quality of PDBM characterised by the occurrence of high microbial counts which may reduce shelf life and can be a reservoir of pathogenic bacteria that potentially pose public health risks in PDBM. A wide diversity of Enterobacteriaceae species belonging mostly to spoilage microbiota was detected in PDBM. Considerable genetic diversity was found among the *E. coli, S. liquefaciens* and *K. oxytoca* strains. Multidrug resistant pathogenic *E. coli* in both raw and pasteurised retail PDBM were detected, which is a cause of concern since these strains are a potential source of antimicrobial resistant foodborne pathogens to humans through the food chain. These findings call for further assessment of the potential risk posed to public health by the milkborne pathogens through quantitative microbial risk assessment as a prerequisite to formulation of situation specific mitigation strategies.

CHAPTER FOUR

EXTENDED-SPECTRUM β-LACTAMASE, SHIGATOXIN AND HAEMOLYSIS CAPACITY OF O157 AND NON-O157 *E. COLI* SEROTYPES FROM PRODUCER-DISTRIBUTOR BULK MILK

Redrafted from:

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4.1. ABSTRACT

The current study investigated virulence genes (*stx1, stx2* and *hlyA*), serotypes and extended-spectrum β-lactamase (ESBL) producing capacity in O157 and non-O157 *Escherichia coli* isolated from producer-distributor bulk milk (PDBM). Fifteen different *E. coli* O-serogroups were observed from the isolates (n=121). The prevalence of *stx1* and *stx2* genes among the *E. coli* isolates was 8.3% and 11.6% (n=121), respectively, while 5.8% harboured both *stx1* and *stx2*. Four *E. coli* isolates (3.3%) had ESBL producing capacity, resisted multiple cephalosporins and astreonam, and carried *stx* genes. Cluster analysis using GTG_5 finger printing revealed a diversity of *E. coli* seropathotypes in PDBM which are known to be associated with human diarrhoeal diseases. These results highlight a potential risk posed on human health by the consumption of PDBM contaminated with pathogenic *E. coli*. A further quantitative risk assessment of the impact of pathogenic *E. coli* contamination in PDBM on human health is therefore recommended.

4.2. INTRODUCTION

In the past few decades numerous studies have targeted *E. coli* O157:H7 serotype in food borne illness outbreaks due to its association with life-threatening diseases (Khan et al., 2002). However, in recent years, the prevalence of emerging non-O157 *E. coli* pathotypes, in particular non-O157 shigatoxin producing *E. coli* (STEC), infections and foodborne diarrhoeal disease has increased (Constantiniu, 2002). The STEC group is very diverse, and has emerged as an important food borne pathogen owing to its insidious strains belonging to a broad range of O & H serotypes that have been implicated in human and animal diseases (Paton & Paton, 1998). Gillespie et al. (2005) reported that the estimated risk of death in STEC outbreaks in European countries was at least five times more than outbreaks attributed to other intestinal pathogens. The frequency of outbreaks and

sporadic cases of infection caused by pathogenic *E. coli* in food is often high also in developed countries despite the implementation of proper public health policies and control measures (CDC, 2005; EFSA, 2015). Continuing epidemiological and clinical reports around the globe on O157 and emerging non-O157 *E. coli* serotypes has emphasised the need for research on these pathogens in foods in order to monitor their incidence and spread in an attempt to protect public health (Constantiniu, 2002).

The public health impact of pathogenic and commensal *E. coli* has also increased over the years due to acquisition of antibiotic resistance genes and subsequent evolution of strains which are resisting commonly used antibiotics (da Silva and Mendonça, 2012). The spread of resistance in *E. coli* has threatened treatment of infections by this pathogen in both humans and animals and is also of increasing public health concern (Allocati et al., 2013). Recently, reports in the US have highlighted unprecedented foodborne outbreaks associated with antibiotic resistant pathogens (CDC, 2013a). *E. coli* is reported among the top six drug resistant microbes requiring urgent development of novel therapies (Shah et al., 2004). Reports of *E. coli* strains being capable of producing Extended Spectrum β-lactamases (ESBLs) and hydrolysing the new generation cephalosporins and aztreonam further necessitates new therapies (Rawat and Nair, 2010). Very little information has been published about Enterobacteriaceae producing ESBLs in milk.

In addition to consequences on human health, the distribution of resistance in *E. coli* pathotypes is associated with genetic mobile elements, such as plasmids, that may also carry virulence determinants (da Silva and Mendonça, 2012). STEC O157 and non-O157 serotypes are characterised by plasmid carrying *stx1*, *stx2* and *eae* virulence factors which may cause severe hemorrhagic colitis (HC) and Haemolytic Uremic Syndrome (HUS) (da Silva and Mendonça,

2012). However, association of virulence and antibiotic resistance in non-O157 pathotypes in particular is underreported (Orden et al., 2000).

Food, especially of bovine origin, is one of the major transmitters of *E. coli* and its pathotypes to humans (De Buyser et al., 2001; CDC, 2005; EFSA, 2015). One of the most incriminated foods of bovine origin which is linked to disease outbreaks around the word is milk and its products (Oliver et al., 2005). In South Africa (SA) a significant population, especially the poor and vulnerable, is supplied with either raw or pasteurised milk directly from producer known as producer-distributor bulk milk (PDBM). This category of milk in SA, though a significant source of income to the farmers, is reported to pose a risk to the public due to pathogens, including *E. coli* (O'Ferrall-Berndt, 2003; Lues et al., 2010; Caine et al., 2014). Very few studies have been reported about characterisation of pathogenic *E. coli* in PDBM. However, a recent study on bulk milk from a province in SA reported diarrheagenic *E. coli* belonging to enteroaggregative *E. coli* (EAEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) and uropathogenic *E. coli* (UPEC) from the milk (Caine et al., 2014). Ntuli et al. (2016), previously identified *E. coli* in PDBM from different geographical regions, which is sold directly from producer to the public in SA. The current study was carried out to characterise 121 STEC O157 and non-O157 strains from PDBM for prevalence and distribution of virulence genes encoding for shigatoxin and haemolysis genes, serogroups and ESBL producing capacity. Genotypic and phenotypic relationship of the *E. coli* isolates was also determined. This was done with the aim of enhancing often limited data, which is important in assessing the risk on human health posed by pathogenic *E. coli* in milk, particularly in developing countries. Information obtained from this study will be used to carry out a quantitative risk assessment of the impact of STEC on public health in PDBM.

4.3. MATERIALS AND METHODS

4.3.1. *E. coli* strains

E. coli isolates (n=121) used in the current study had previously been isolated from raw and pasteurised PDBM from different provinces in South Africa and identified using MALDI-TOF (Chapter 3).

4.3.2. Screening for β-lactamase producing *E. coli*

ESBLs producing *E. coli* isolates were first screened using a disc diffusion test with extended-spectrum cephalosporins (ceftriaxone (CRO 30 μg), cefpodoxime (CPD 10 μg), amoxiclav (AMC 30 μg), ceftazidime (CAZ 30 μg), cefotaxime (CTX 30 μg)) and aztreonam (ATM 30 μg) (Bio-Rad Laboratories, Hercules, CA, USA) on Mueller-Hinton agar II (Smet et al., 2008; Njage and Buys, 2015). *E. coli* isolates which showed resistance (CLSI, 2011) to any one of the six antibiotics were further screened for phenotypic production of ESBLs. Confirmation of ESBLs producing *E. coli* isolates was done using the modified double disc diffusion method on Mueller-Hinton agar II (Pitout et al., 2003). Cefotaxime + clavulanic acid (30 μg + 10 μg), ceftazidime + clavulanic acid (30 μg + 10 μg), amoxicillin + clavulanic acid (30 μg + 10 μg) discs (Bio-Rad Laboratories) were used. Extended-spectrum β-lactamase was positive when the zone diameters given by the discs with clavulanate were ≥ 5 mm larger than those without the inhibitor for at least one of the combinations. *E. coli* ATCC 25922 (ESBL negative control), *E. coli* ATCC 35218 (ESBL positive control) were used in the study (Njage and Buys, 2015). Data for antibacterial agent susceptibility tests performed during screening for ESBLs were recorded as zone diameters (mm) and a hierarchical cluster analysis was performed using Euclidean distance matrix with un-

weighted pair group method and arithmetic means using XLSTAT version 2014.08.09 (www.**xlstat**.com).

4.3.3. Extraction of genomic DNA

Extraction of DNA for all the PCR analyses was done as follows: Bacterial strains were grown in 2 ml tryptone soy broth and incubated at 37 ºC for 24 h. The cultures were harvested by centrifugation at 9184 rcf (g) for 10 min. Each bacterial pellet was resuspended in 200 µl of lysis buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 % Triton X-100 (SIGMA, St. Louis, MO, USA)) (Goldenberger et al., 1995). The mixture was incubated at 95 ºC for 30 min with agitation. This was then cooled to 4 ºC for 10 min and centrifuged at 13000 rpm for 10 min. The DNA containing supernatant was purified using phenol chloroform method as described by Wilson (2001). DNA concentration was measured using a NanoDrop (Jeneway Genova Nano, Staffordshire, UK) and the concentration was standardised to 100 ng/µl. DNA extracts were stored at 4 ºC before use. *E. coli* isolates were confirmed using PCR detection of the *uidA* gene (Bej et al., 1991). All the *E. coli* isolates were characterised using primers and PCR thermal cycle conditions depicted in Table 4.1.

Table 4.1: Oligonucleotide primers used in the study for detection of targeted genes of *E. coli* isolated from producer-distributor bulk milk and the different thermal cyclic conditions applied.

Target Gene	Primer name	Sequence (5' - 3')	Fragment size bp	Source	PCR cycling conditions
Alpha-haemolysin	*hlyA F*	GTCTGCAAAGCAATCCGCTGCAAATAAA	561	(Kerényi et al., 2005)	**35 X[a]** 95 ºC for 60s, 54 ºC for 60s, 72 ºC for 60s
	hlyA R	CTGTGTCCACGAGTTGGTTGATTAG			
*uid*A	*uidA F*	GGTCACTCATTACGGCAAAG	379	(Bej et al., 1991)	**30 X[b]** 94 ºC for 60s, 59 ºC for 60s, 72ºC for 60s
	uidA R	CAGTTCAACGCTGACATCAC			
Shigatoxin 1	*SLT-1LP 30*	CAGTTAATGTGGTGGCGAAGG	348	(Cebula et al., 1995)	**35 X[c]** 94 ºC for 30s, 58 ºC for 45s, 72 ºC for 90s
	SLT-1LP 31	CACCAGACAATGTAACCGCTG			
Shigatoxin 2	*SLT-2LP 43*	ATCCTATTCCCGGGAGTTTACG	584	(Cebula et al., 1995)	**35 X[c]** 94 ºC for 30s, 58 ºC for 45s, 72 ºC for 90s
	SLT-2LP 44	GCGTCATCGTATACACAGGAGC			
Mutated *uid*A for *E. coli* O157:H7	*uidA PT-2*	GCGAAAACTGTGGAATTGGG	252	(Cebula et al., 1995)	**35 X[c]** 94 ºC for 30s, 58 ºC for 45s, 72 ºC for 90s
	uidA PT-3	TGATGCTCCATAACTTCCTG			
rfb gene cluster	*JUMPstart*	CACTGCCATACCGACGACGCCGATCTGTTGCTTGG	1500 -1700	(Coimbra et al., 2000)	As explained in the method
	gnd	ATTGGTAGCTGTAAGCCAAGGGCGGTAGCG			

*The 2X ReadyMix contains KAPA Taq DNA Polymerase (1 U per 50 µl reaction), KAPA Taq Buffer, dNTPs (0.2 mM of each dNTP at 1X), $MgCl_2$ (1.5 mM at 1X) and stabilisers (Boston, MA, USA). PCR grade water (KAPA bio-systems, Salt River, CPT, SA). Unless stated, all PCR products were electrophoresed (120 V for 45 min) in 1.5 % agarose gels, stained with GelRed (Biotium, CA, USA) and visualised using Gel documentation system (Gel Doc EZ Imager, BioRad, Hercules, CA, USA). A GeneRuler 1 kb DNA ladder (Thermo Scientific, Waltham, MA, USA) was used as a size marker. All PCR reactions were done using the thermocycler (C1000 Touch ThermalCycler CFX96TM Real Time System (Bio-Rad). [a]Initial denaturation step of 95 ºC for 15 min and final strand extension steps of 72 ºC for 7 min. [b,c]Initial denaturation step of 95 ºC for 3 min and final strand extension step of 72 ºC for 8 min.

4.3.4. Haemolysis on blood agar

Columbia agar supplemented with 5% (v/v) sheep blood (Bio-Rad, Marnes-la-Coquette, France) was used to identify haemolysis activity in the *E. coli* isolates. Indication of alpha-haemolysin and all haemolysin types (beta and gamma) were examined after 6 h and 24 h respectively (Schmidt and Karch, 1996). To test for the presence of the *hlyA* gene, PCR was then performed on the isolates which had given positive results for alpha-haemolysis activity (Lorenz et al., 2013). The 20 µl reaction mixture consisted of 1 µl (100 ng) DNA template, 2 µl (10 µM) of each *hlyA* primers (Table 4.1), 10 µl of 2 times KAPA Taq ReadyMix PCR Kit and PCR grade water.

4.3.5. *E. coli* O157:H7 and virulence gene determination

A multiplex PCR assay using three sets of primers targeting *stx1*, *stx2* and *uidA* genes was used to identify *E. coli* O157:H7 and shigatoxin virulence genes (Cebula et al., 1995). For the *uid*A gene, mismatch amplification mutation assay (MAMA) primers, specific for a unique base substitution in *uid*A of *E. coli* O157:H7, were used (Table 4.1). *E. coli* O157:H7 (ATCC 35150) was used as a positive control. The 20 µl reaction mixture consisted of 1 µl (100 ng) DNA template, 0.5 µl (10 µM) of each *stx1*, *stx2* and *uid*A primers, 10 µl of 2 times KAPA Taq ReadyMix PCR Kit and PCR grade water.

4.3.6. GTG_5 Repetitive extragenic palindromic (REP)-PCR fingerprinting of the *E. coli* isolates.

Fingerprints were generated for the 46 *E. coli* isolates including the O157:H7 reference strain. The 46 fingerprinted isolates had previously given a positive presumptive test on STEC media (Chapter 3). The 20 µl reaction mixture consisted of 2 µl (100 ng) DNA template, 0.3 µL (0.2 µM) of the

single oligonucleotide $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) primer (Versalovic et al., 1994), 10 µl of 2 times KAPA Taq ReadyMix PCR Kit, 0.4 µl (1%) dimethylsulphoxide and PCR grade water. The PCR thermal cycling was performed as follows: Initial denaturation at 95 ºC for 10 min; 35 cycles at 95 ºC for 30 s; 40 ºC for 60 s and 65 ºC for 3 min and a final elongation step at 65 ºC for 8 min. The PCR amplified DNA was separated on 2% agarose gel (55 V for 4 h). The GTG_5 fingerprints of the *E. coli* isolates were analysed using GelCompar II version 6.5 (Applied Maths NV, Sint-Martens-Latem, Belgium). The similarity of digitised bands patterns was calculated using Pearson's correlation coefficient, and the unweighted pair group method with arithmetic means. Complete linkage algorithms were used to construct an average linkage dendrogram to show relationship of isolates. Isolates were considered to be within a clonal cluster if relatedness was 70% and above (Stackebrandt et al., 2002).

4.3.7. O-Serotyping

A method by Coimbra et al. (2000) was modified and used for serotyping the *E. coli* isolates. The O-antigen gene cluster (*rfb*) was amplified prior to restriction digestion using restriction enzyme *Mbo*II. JUMPstart and *gnd* primers (Wang and Reeves, 1998) were used (Table 4.1) to amplify the O-gene cluster in *E. coli*. A touchdown PCR cycle was done as follows: denaturation at 94 °C for 10 s, annealing at 64 °C for 30 s, and extension at 68 °C for 15 min, repeated ten times. For the next 20 cycles, extension time was increased by 20 s each time. Initial denaturing step (94 °C for 2 min) and final elongation (72 °C for 7 min) steps were included. Amplicons were electrophoresed in 1.5% agarose gels and their sizes were estimated using a GeneRuler 1 kb plus DNA ladder (Thermo Scientific, Waltham, MA, USA). In cases where multiple bands were obtained, the band with the highest molecular weight within size range 0.5 to 1.7 kbp was isolated and purified using an E-Gel® Electrophoresis System (E-Gel® Safe Imager Real-time Transilluminator (Invitrogen,

Carlsbad, CA, USA)) with E-Gel® Precast Agarose (2%) (Invitrogen) prior to restriction digestion.

Restriction analysis was as follows: 10 µl of the PCR product was mixed with 1 µl of *Mbo*II (FastDigest enzyme), 17 µl of nuclease free water and 2 µl of 10 X FastDigest buffer (Thermo Scientific, Waltham, MA, USA). After incubation at 37 ºC for 5 min the enzyme was denatured at 65 ºC for 5 min. The restriction product was then electrophoresed in 2% agarose at 55 V for 5 h. Gel images were taken under UV illumination. BioNumerics software version 6.0 (Applied Maths NV, Sint-Martens-Latem, Belgium) was used to interpret the Restriction Fragment Length Polymorphism (RFLP) patterns by construction of a dendrogram based on the patterns. A dendrogram was constructed on the basis of a binary table in which the presence or absence (1/0) of restriction fragments was recorded. The "Ward" algorithm of the R-routine "hclust" was used (BioNumerics, Applied Maths, Sint-Martens-Latem, Belgium). A 1.5% band position tolerance was applied for comparison of RFLP fingerprinting patterns (Karger et al., 2011). Isolates were considered to be within a clonal cluster if relatedness was 70% and above (Stackebrandt et al., 2002). Representative isolates within a cluster were sent for serotyping using agglutination assays at an ISO 17025 certified national reference laboratory (Agricultural Research Council-Onderstepoort Veterinary Institute, Pretoria, SA).

4.4. RESULTS

4.4.1. Virulence genes and ESBLs in *E. coli*

Resistance to cephalosporins (ceftriaxone (CRO), cefpodoxime (CPD), amoxiclav (AMC), ceftazidime (CAZ), cefotaxime (CTX)) and aztreonam (ATM) used during screening of ESBLs

was detected in all the *E. coli* isolates tested. While a total of 97.5% of the isolates were susceptible to cefotaxime, cefpodoxime was the most frequently resisted antibiotic (66.1%) (Figure 4.1a).

Resistance patterns to cephalosporins and aztreonam revealed six different antibiotic resistance phenotypes (resistant to either a single or a combination of cephalosporins and aztreonam) and resistance to cefpodoxime gave the highest frequency (24.8%) of phenotypes (Figure 4.1b). Resistance to cefpodoxime was observed to be common in all the phenotypes resistant to multiple cephalosporins and aztreonam, and ATM-CPD was the phenotype combination with the highest frequency (14.0%, n=121) (Figure 4.1b). Four (3.3%) *E. coli* isolates belonging to serogroups O2, O9 and O68 had ESBL producing capacity, resisted multiple cephalosporins and aztreonam, and also carried either *stx1* or *stx2*. Out of the 121 *E. coli* isolates, 31 (25.6%) harboured shigatoxin virulence genes. A total of 8.3% (n=121) carried *stx1* genes and 11.6% were positive for *stx2*, while 5.8% exhibited both (Table 4.2). Five *E. coli* isolates from the milk were positive for alpha-haemolysis (two O9, one O83 and two O112) on sheep blood agar and only 3 (one O9 and two O112) carried the *hlyA* gene (Table 4.2). The isolates which carried the *hlyA* gene also exhibited either *stx1* or *stx2* genes. A total of 25 (20.6%, n=121) *E. coli* isolates were capable of producing ESBLs. Eight of the serotypes (one O2, two O9, one O20, one O68 and three O157) which produced ESBLs also carried either one or a combination of *stx1* and *stx2* genes (Table 4.2).

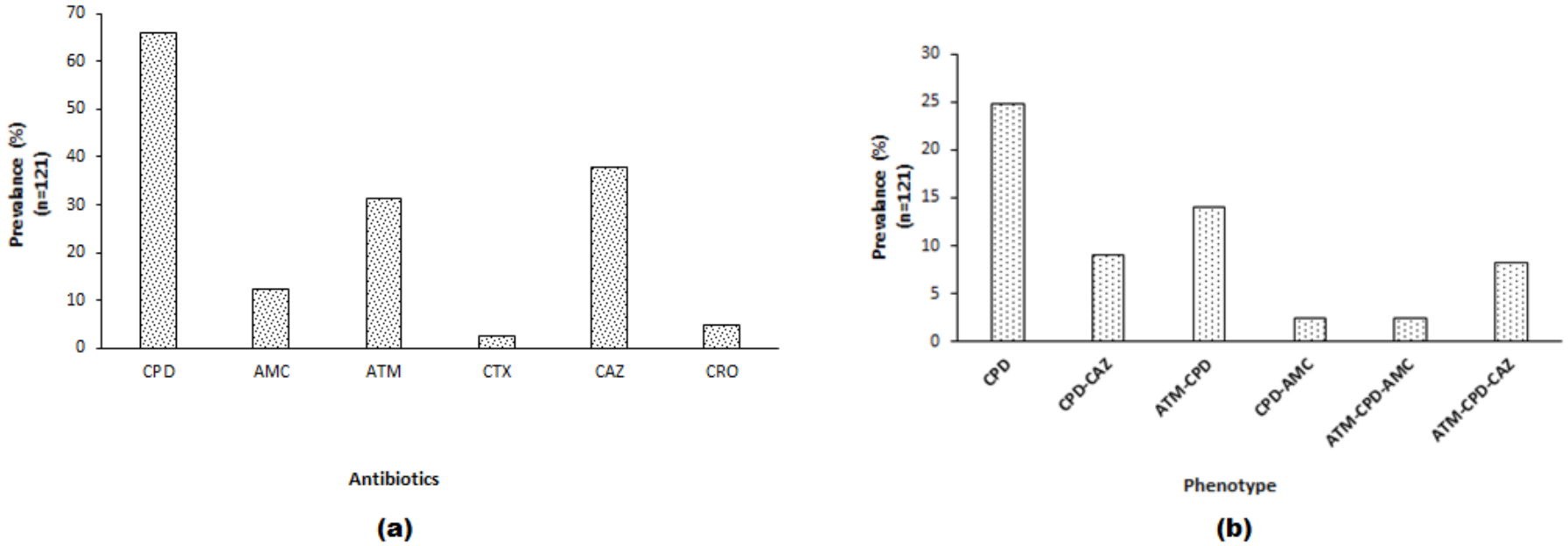

Figure 4.1: Prevalence of: (a) *E. coli* isolates resistant to different cephalosporins and aztreonam; (b) resistant *E. coli* phenotypes isolated from producer-distributor bulk milk.

*The cephalosporins and aztreonam were used to screen for ESBL producing *E. coli*.

*(CRO – Ceftriaxone, ATM – Aztreonam, CPD – Cefpodoxime, AMC – Amoxiclav, CAZ – Ceftazidime, CTX – Cefotaxime).

4.4.2. Serotyping (O-gene cluster and restriction analysis)

Amplification of the O-gene cluster generated one amplified fragment which ranged in size from 0.2 – 2 kbp. After *MboII* digestion, clear bands were obtained for most of the serogroups. The number of bands per pattern varied from 4 to 10 (Figure 4.2).

Cluster analysis of RFLP patterns revealed 16 clusters at 75% similarity level (Figure 4.3). A total of 32 representative isolates; 2 from each sub-clusters were sent for serum agglutination assay. The assumption drawn was that clonal strains clustered at ≥75% similarity. The dendrogram revealed that out of the 121 *E. coli* strains from the milk, 90 (74.2%) strains were associated with 15 different O-serogroups and 31 (25.6%) were considered rough strains (Figure 4.3). Rough *E. coli* strains were untypeable isolates with serum agglutination due to auto-agglutination with all the O-antisera used. The dominant and most frequently identified O-serotype were O9 (19.8%), followed by O157 (14.0%) serogroup, while O4 (0.8%), O43 (0.8%) and O119 (1.7%) had lower prevalence (Figure 4.4). A total of 26.6% of the serotypes (O2, O9, O64, O157 and Rough) were associated with both pasteurised and raw PDBM while the rest of the serotypes were identified in raw milk only.

4.4.3. Cluster analysis of *E. coli* serotypes in terms of phenotypic and genotypic relationship

In order to establish the phenotypic and genotypic relationship of *E. coli* isolates according to serogroup, the clustering trends on dendrograms generated from antibiotic susceptibility data (used during screening for ESBLs) and GTG_5 finger prints, were observed.

Table 4.2: Number of *E. coli* strains with virulence genes and/or ESBL producing capacity for O157 and non-O157 STEC isolates from producer-distributor bulk milk.

Virulence gene(s) and/or ESBL producing capacity	**Serotype**																**Total** (%)
	O2	**O4**	**O9***	**O20**	**O43**	**O64**	**O68**	**O83***	**O85**	**O109**	**O112***	**O119**	**O147**	**O155**	**O157**	**Rough**	
*hly*A (only)	-	-	1	-	-	-	-	-	-	-	2	-	-	-	-	-	**3** (2.45%)
Stx1 (only)	1	-	1	1	1	1	-	-	-	-	1	-	-	-	4	-	**10** (8.3%)
Stx2 (only)	-	-	3	-	-	1	1	-	-	-	-	-	-	1	8	-	**14** (11.6%)
Stx1 and *Stx2* (only)	-	-	-	-	-	-	-	1	-	-	1	-	-	-	5	-	**7** (5.8%)
ESBLs (only)	-	-	7	1	-	1	1	-	-	-	-	-	2	1	-	4	**17** (14.0%)
ESBLs and *Stx1/Stx2*	1	-	2	1	-	-	1	-	-	-	-	-	-	-	3	-	**8** (6.6%)

* (-) not detected, ESBLs – Extended Spectrum β-lactamases, *Stx* – shigatoxin gene, *hly*A – haemolysis gene. Serotypes with asterisk (*) were positive for alpha haemolysis on sheep blood agar (two O9, one O83 and two O112).

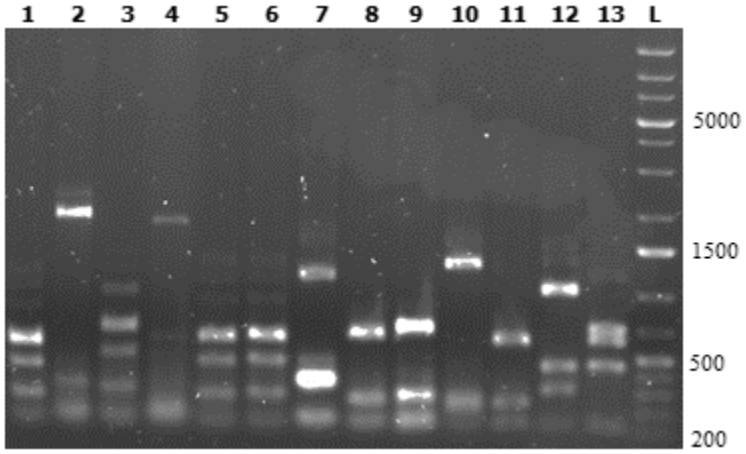

Figure 4.2: Illustrative agarose gel electrophoresis image of DNA fragments after *MboII* digestion.

*Lanes 1= O9, 2= O43, 3= O9, 4= O4, 5= O9, 6= O9, 7= O112, 8= O64, 9= O64, 10= O155, 11= O64, 12= O147, 13= O64, L= molecular weight maker.

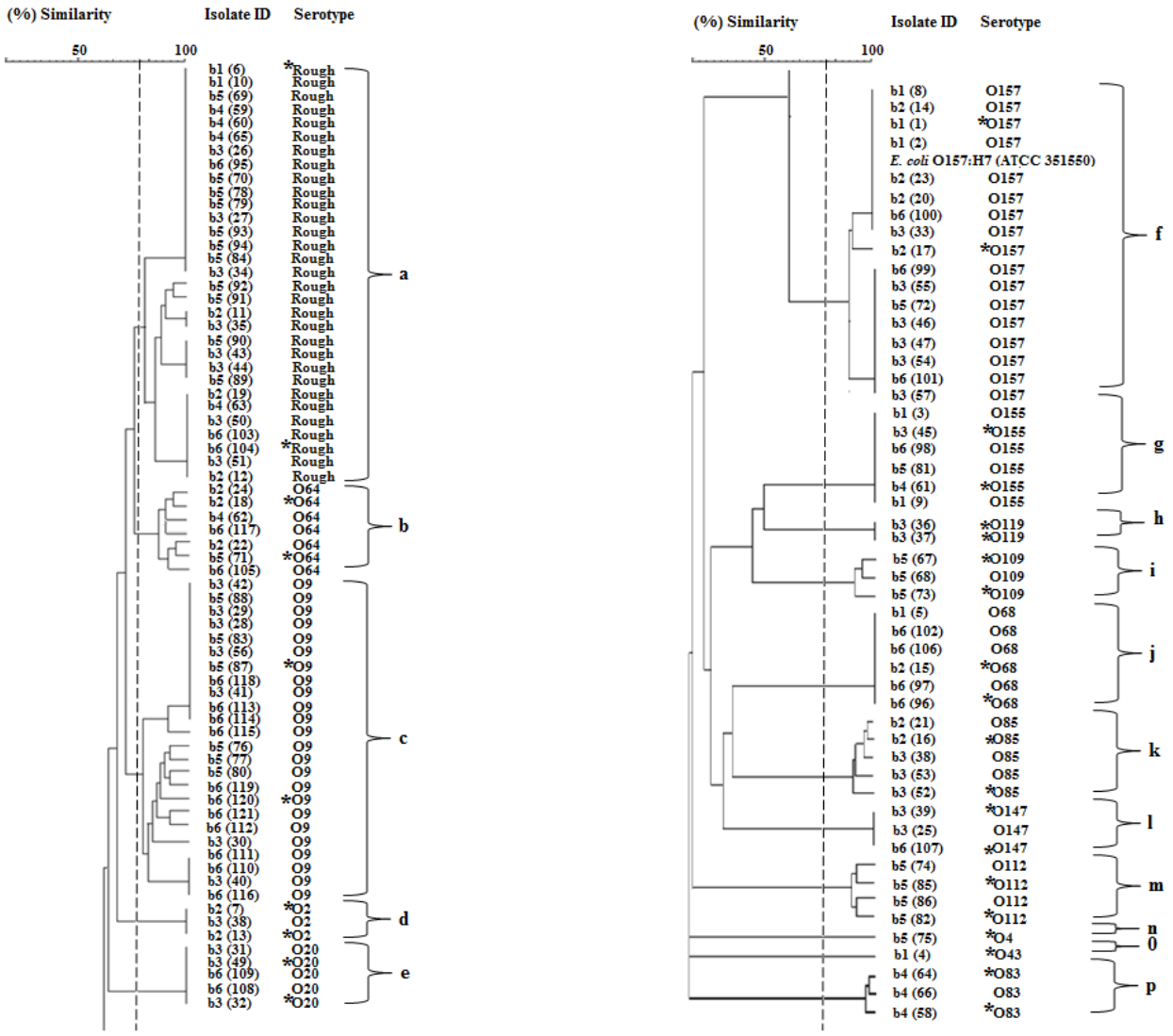

Figure 4.3: Dendrogram for RFLP fingerprints of *E. coli* isolates obtained from producer-distributor bulk milk.

*Clusters defined at ≥75% similarity. Isolates within a cluster in asterisks (*) were sent for serotyping using serum agglutination assays at an ISO 17025 certified national reference laboratory (Agricultural Research Council-Onderstepoort Veterinary Institute, Pretoria, SA).

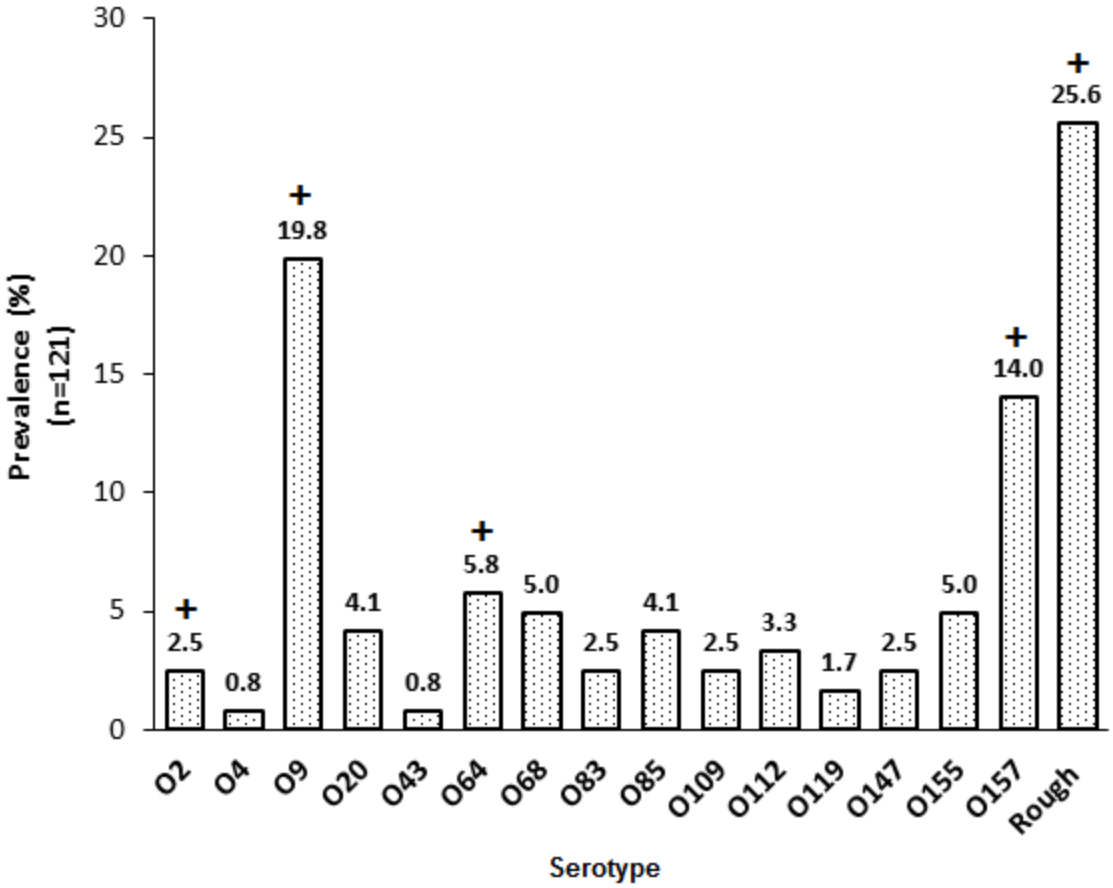

Figure 4.4: Prevalence of *E. coli* serotypes isolated from producer-distributor bulk milk (n=121).

**E. coli* serotypes with plus sign (+) at the top were isolated from both raw and pasteurised retail bulk milk samples whereas the rest were isolated from raw milk only. Rough *E. coli* strains were untypeable isolates with serum agglutination due to auto-agglutination with all the O-antisera used.

Cluster analysis was applied to 25 *E. coli* isolates which produced ESBLs. This was done to determine trends in common phenotypes and relationship of isolates according to serogroup. Figure 4.5 presents six major clusters (A – F), at 75% similarity, that were further analysed for patterns of association of *E. coli* isolates. There was no trend of clustering which was observed amongst the ESBLs *E. coli* isolates, though serotype O9 was distributed in most of the clusters. Cluster group E was dominated by serotype O9 (66.6%). The common phenotypes in clusters C,

E and F were resistant to CPD-CAZ, ATM-CPD and ATM-CPD-CAZ respectively. In general, clustering of isolates on the basis of antibiotic resistance phenotypic characteristics revealed no relationship of the *E. coli* isolates based on serogroup. However, a genetic relatedness of 31 STEC and 15 non STEC isolates using rep-PCR fingerprints revealed clustering which was based on serogroups (Figure 4.6).

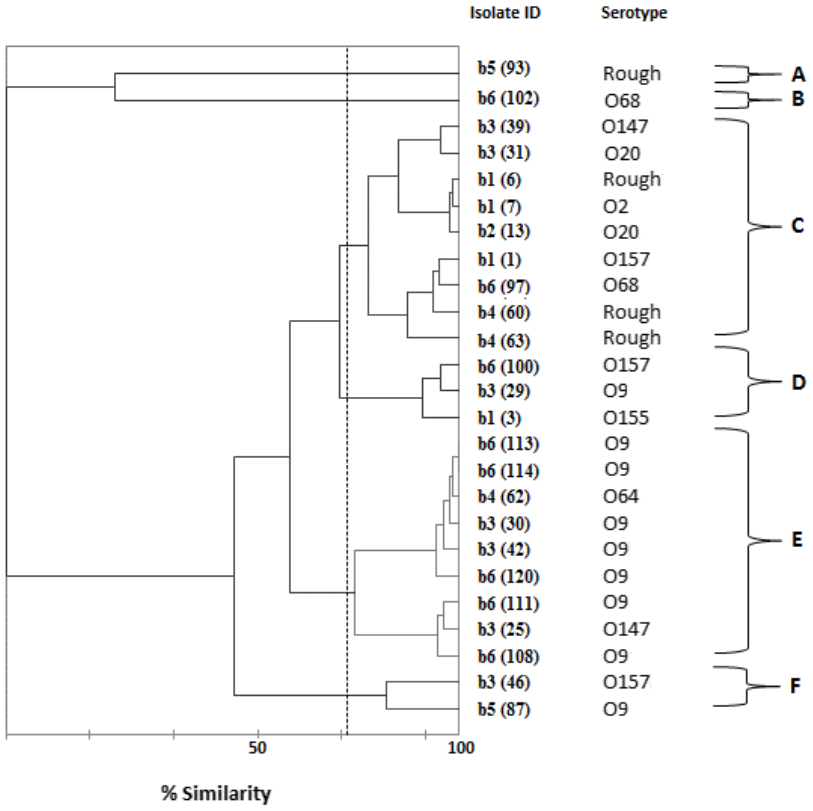

Figure 4.5: Two-dimensional hierarchical cluster analysis dendrogram showing relationship amongst 25 extended beta-lactamase spectrum (ESBL) producing *E. coli* isolated from producer-distributor bulk milk.

*Clusters defined at ≥75% similarity. The dendrogram was based on inhibition zone diameter data for antibiotics used for screening ESBL producing *E. coli*.

Figure 4.6 presents seven clusters which were generated from the dendrogram. The largest cluster (i) was dominated by serogroup O9 followed by O83 and O112. Further analysis of the cluster revealed that isolates were also grouping based on *stx* and *hlyA* genes. There was no common antibiotic resistance patterns observed in this cluster. The second largest cluster (v) comprised of serotype O157. Relatedness of these isolates was based on genotypic and phenotypic characteristics. The isolates that grouped in this cluster had been found to have common antibiotic phenotypic patterns (resistant to CPD and CPD – CAZ). Seventy percent of serotypes in cluster (v) carried *stx 2* genes. Cluster groups (ii), (iv), (vi) and (vii) were unique for serotype O157, O157, O9 and O20, respectively. In addition, common resistance to CPD had also been recoded for isolates that grouped in cluster (vii).

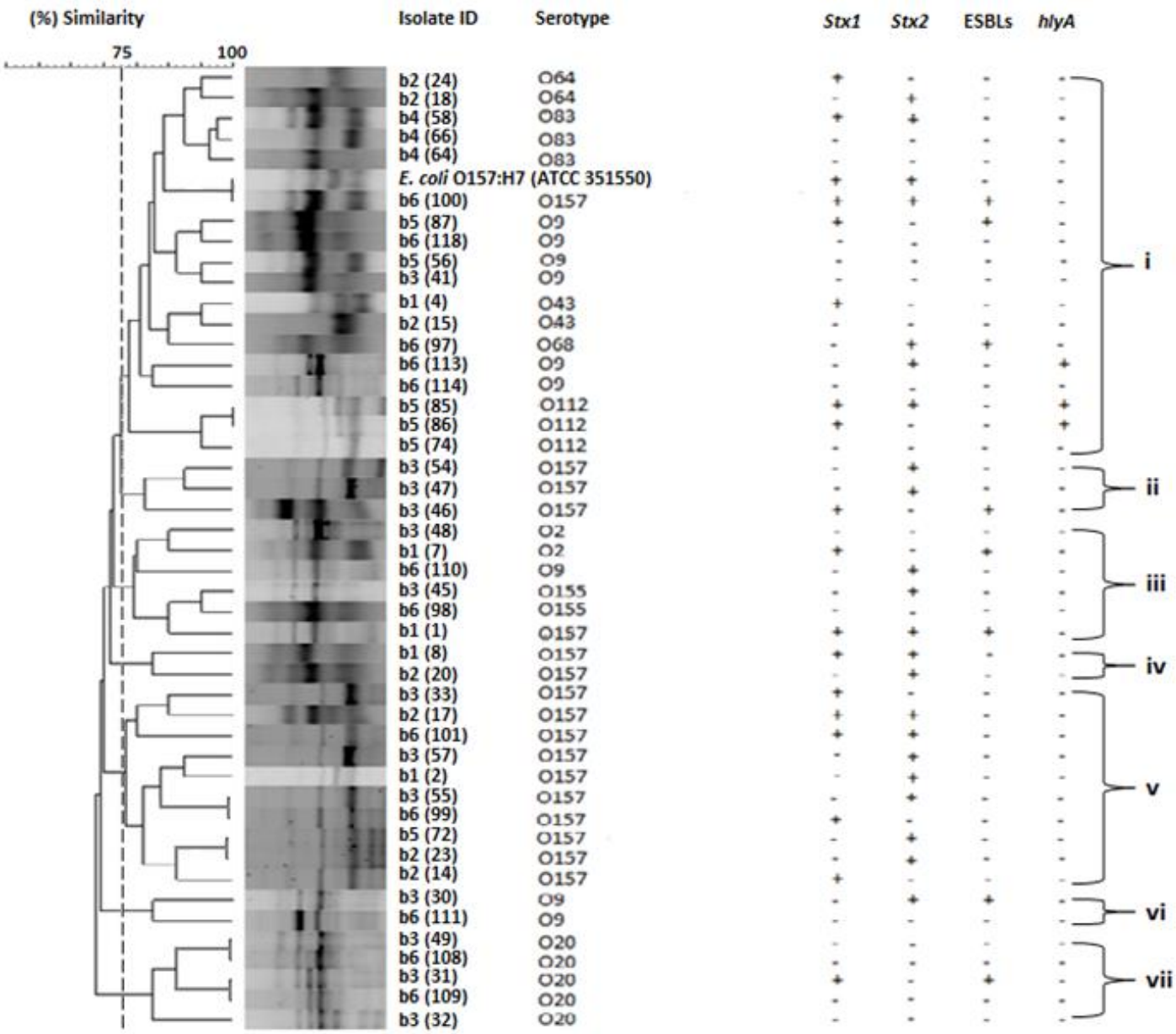

Figure 4.6: Dendrogram for rep-PCR fingerprints showing the relationships based on serotype, and virulence profiles amongst 46 isolates from retail producer-distributor bulk milk.

*****Clusters defined at ≥75% similarity.

4.5. DISCUSSION

This current study detected multiple resistant *E. coli* to cephalosporins and aztreonam during screening for ESBLs producing *E. coli*, with 20.6% of the isolates to be phenotypically positive for ESBLs production (Figure. 4.1; Table 4.2). Reports on acquisition of antimicrobial resistance genes in *E. coli*, and emergence of new strains capable of hydrolysing new generation cephalosporins, have escalated over the years around the globe (da Silva and Mendonça, 2012). However, acquisition of antimicrobial resistance genes by bacteria in environments such as food has not been studied extensively (Straley et al., 2006). This probes for more research work in this area of study. Again, very few studies are available on the presence of ESBLs Enterobacteriaceae in milk (Hammad et al., 2008; Geser et al., 2012). However, studies carried out in different countries around the world have isolated ESBLs Enterobacteriaceae in dairy cattle, livestock and vegetables (Girlich et al., 2007; Watson et al., 2012; Njage and Buys, 2015). ESBLs genes are plasmid-mediated and molecular detection of the genes is recommended as plasmids have been implicated in the spread of resistance. Rough *E. coli* strains in this study produced ESBLs. Even though rough strains are mostly non-pathogenic, this is a cause of concern since commensal *E. coli* are reported as reservoirs of antibiotic resistance genes and can transfer these genes, through lateral gene transfer, to the pathogenic strains (Straley et al., 2006). In the current study, 66.1% of *E. coli* were resistant to cefpodoxime. Resistance to cefpodoxime has been reported to be mediated by a mutation in the attenuator or the weak promoter of the chromosomal *ampC β-lactamase* gene and this leads to enhanced production of AmpC, resulting in resistance to extended-spectrum cephalosporins (Caroff et al., 1999).

STEC seropathotypes with different combinations of virulence genes and ability to cause haemolysis were identified (Table 4.2). Similar observations to this present study, on prevalence

of STEC with different combinations of virulence genes, have been reported for STEC in milk (Rey et al., 2006; Altalhi and Hassan, 2009). Thirty one *E. coli* isolates in this study harboured either single or a combination of *stx1* and *stx2* genes and this was prominent in the O157 serogroup. A total of 74.4% *E. coli* isolates in this study did not harbour the *stx* gene. However, other virulence genes, which were not tested for in the current study but known to cause diarrheagenic diseases, could be present in these isolates. A survey by Caine et al. (2014) on raw milk from two commercial dairy farms in SA did not detect *stx* (n=100) from *E. coli* isolates. However the authors detected *fliC*H7 gene (43%, n=100) used as an identification of *E. coli* O157:H7 serotype and also other virulence factors in *E. coli* associated with EPEC, EAEC, ETEC and UPEC. Therefore, to fully understand the risk posed to the public by consumption of PDBM, a comprehensive study on the virulence factors of diarrheagenic *E. coli* in recommended. Similar distribution patterns of *stx* genes (*stx1* (19%) and *stx2* (20.8%) have been reported in studies on *E. coli* O157:H7 in milk and dairy cows (Hoffmann et al., 2014). STEC non-O157 serogroups (O2, O4, O9, O20, O43, O64, O68, O83, O85, O109, O112, O119, O147, O155) reported in this study, were different in comparison to commonly isolated STEC non-O157 (O111, O26, O103, and O145) serogroups from foods such as milk in continental Europe (Eklund et al., 2001). The dominant *stx* virulence gene in *E. coli* O157:H7 is *stx2* as reported by Khan et al. (2002) and this was also evident in this current study. This high proportion of *stx2* in *E. coli* from PDBM poses an important risk for consumers since the gene is reported to produce the most virulent Shigatoxin for humans compared to *stx1* (Karch et al., 2005).

In the present study, 4.1% of the *E. coli* isolates were phenotypically haemolytic and the *hlyA* gene was only present in 2.4% of the isolates (Table 4.2). *E. coli*, in the current study, which exhibited haemolytic expression on blood agar could have carried other hemolysin genes such as *sheA*, *ehxA*,

and *e-hlyA* (Kerényi et al., 2005*)*. In this study, genotypic and phenotypic enterohaemolysin activity in non-O157 *E. coli* was detected. The presence of alpha-hemolysin gene is often a characteristic of *E. coli* belonging to serogroup O157 and is less frequently associated with STEC of other serogroups particularly noted in the present study.

A total of 3.3% *E. coli* isolates in the current study had a combination of ESBLs, multiple resistance to cephalosporins and aztreonam and *stx* virulence factors. A review study by da Silva and Mendonça (2012) revealed an association between antibiotic resistance and virulence factors in *E. coli*. Rasko et al. (2011) reported that acquisition of genes in *E. coli* through lateral gene transfer can result in an accretion of synergistic antibiotic resistance and virulence factors. Bacteria subjected to antibiotic selection pressure can reduce genetic barriers between distantly related bacteria resulting in a rapid emergence of antibiotic resistance and the import of virulence genes by lateral gene transfer (Wirth et al., 2006). Presence of pathogenic *E. coli* in this study, which was antibiotic resistant, is of considerable importance from the standpoint of public health since it reduces the effectiveness of medicines. Outbreaks caused by pathogenic *E. coli* in food/milk have been reported (CDC, 2005; EFSA, 2015).

In this study, a method by Coimbra et al. (2000) was adopted and modified, which integrated genotypic and phenotypic data for serotyping *E. coli* isolates in this study. The method demonstrated cost effectiveness and was less time intensive and improves the possibility of detecting the often minimal amount of variation between strains. Sizes of amplified *rfb* gene region fragments for the isolates which ranged from 0.2 – 2 kbp (Figure 4.2) were far lower than what was reported by Coimbra et al. (2000). This could be as a result of the plasticity of O-antigen genes in *E. coli* and more so, the diversity of the genetic pool in different geographical regions (Aydanian et al., 2011). Restriction digestion using *MboII* generated O-patterns with less than 30

band fragments. This is ideal even for visual inspection in case where computer software are not available (Collins and Ross, 1984). Han et al. (1991) reported that large numbers of DNA fragments generated by restriction endonucleases are difficult to interpret, especially when the bands are densely distributed and need complicated computer software. In the current study, similarities and differences in O-patterns among different strains were clear and easily determined by a computer software (BioNumerics software version 6.0).

Cluster analysis of *E. coli* isolates, based on RFLP data (Figure 4.3), grouped some genetically different but phenotypically similar *E. coli* strains into unique clusters. *E. coli* can lose or gain genetic mobile elements through lateral gene transfer (da Silva and Mendonça, 2012). Some of the isolates in the unique clusters had been found to carry *stx* virulence genes. *E. coli* serotypes with either *stx1* and/or *stx2* genes and different antibiotic profiles produced identical O-antigen RFLP patterns and clustered together. This is important for epidemiological and clinical purposes as variations between *E. coli* pathotypes can be detected (Khan et al., 2002). Conventional serotyping using serum agglutination assays poses problems of cross-reaction (i.e. reaction of one O-antigen with O-antisera developed against another O-antigen) between O-serogroups of *E. coli* (Harrigan and McCance, 2014) and the method used, in this study, is more reliable since the indistinctness is resolved. Unlike conventional serotyping, the method used in the current study, also uses genotypic in addition to the phenotypic characteristics. In this study, 25.6% (Figure 4.4) of *E. coli* isolates were untypeable using serum agglutination due to auto-agglutination with all the O-antisera used and were considered rough strains. Rough *E. coli* strains do not produce the O-antigen as a result of mutation within the *rfb* gene clusters (Coimbra et al., 2000). This study did not detect *stx* or haemolysis activity from the rough strains. However, Menrath et al. (2010), Eklund et al. (2001) reported rough *E. coli* strains harbouring *stx 2*, EHEC-*hly A* and *eae* virulence

factors and the strains were isolated from diseased humans with HUS and thrombotic-thrombocytopenic purpura (TTP).

Previous studies on *E. coli* in milk focused more on the eminent O157 serogroup (Upton and Coia, 1994; Bielaszewska et al., 1997; Keene et al., 1997). However, in the current study, attention was on both O157 and non-O157 serogroup in both raw and pasteurised PDBM. Non-O157 STEC are increasingly recognised as an important human pathogen, responsible for several outbreaks (Jacob et al., 2013) and both the European Food Safety Authority (EFSA) and the U.S. Department of Agriculture (USDA) have issued recommendations for laboratory testing for these pathogens (Eblen, 2007; EFSA, 2007). Serogroups O9 and O157 were dominant among the 121 *E. coli* isolates in this study (Figure 4.4). The current study identified 15 different O-serotypes and these results were similar to Zweifel et al. (2010) who observed 13 different O-serotypes of *E. coli* in raw milk. Similar serotypes found in the present study have previously been isolated from cattle and humans; of which 64.0% of the serotypes in the present study were commonly isolated from humans; which suggest cross contamination during the milk chain (Constantiniu, 2002).

This study clustered the *E. coli* isolates using phenotypic and genotypic data in a bid to understand relatedness of the isolates. Phenotypic clustering (based on antibiotic susceptibility data) showed no trend of the *E. coli* isolates clustering according to serogroup. *E. coli* isolates within the clusters which had common antibiotic resistance patterns were observed (Figure 4.5). This particular clustering can be as a result of localised selection pressure from the characteristic antimicrobial agents commonly used as per particular geographical province (Parveen et al., 1999). Owing to the above explained phenomenon, antibiotic resistance profiles have been used in bacterial source tracking in different studies (Scott et al., 2002; Meays et al., 2004).

GTG_5-clustering (Figure 4.6) in this study showed a different trend among the *E. coli* isolates compared to the clustering that was identified using phenotypic data. REP-cluster analysis of the *E. coli* isolates showed a particular trend with respect to serogroups. *E. coli* belonging to the same serogroups have the same DNA regions that are flanked by conserved sequence which generate similar genetic fingerprints when GTG_5 technique is employed (Nürnberg et al., 1989). Studies have shown that cluster analysis using rep-PCR fingerprints (GTG_5) groups *E. coli* isolates according to common ancestry (Aijuka et al., 2014; Njage and Buys, 2015). *E. coli* isolates in the present study were genetically diverse and such degree of genetic diversity has been associated with versatility and genomic plasticity in *E. coli* from either food or the environment (Van Elsas et al., 2011). Cluster analysis of *E. coli* isolates using GTG_5 fingerprints revealed 7 groupings which were based on serogroups in some of the clusters (Figure 4.6) and this supported the earlier articulated clustering results from RFLP fingerprinting (Figure 4.3) which was used in serotyping in this study. Thus, REP-PCR finger prints can also be used in serotyping following the procedure outlined in this study. However, given that grouping of the same serotypes into different clusters and different serotypes into unique serogroups can also occur, use of GTG_5 fingerprinting requires caution.

4.6. CONCLUSION

The results obtained in the current study revealed a diversity of *E. coli* seropathotypes (with different shigatoxin virulence factors and ESBLs producing capacity) known to be associated with human diarrhoeal diseases in PDBM. Isolation of *E. coli* serotypes carrying virulence genes that are known to cause human diseases and are also resistant to antibiotics can present a significant public health risk, especially to the vulnerable members of the community. In the present study, serotypes that are commonly isolated from humans were detected and this suggest cross

contamination during the milk chain. This study highlights a potential risk posed to human health by consuming PDBM contaminated by pathogenic *E. coli*. Further quantitative risk assessment of the impact on human health by pathogenic *E. coli* contamination in PDBM is recommend in order to formulate appropriate food safety objectives and adequate levels of protection that can manage the risk and meet public health goals.

CHAPTER FIVE

QUANTITATIVE RISK ASSESSMENT FOR SHIGATOXIN PRODUCING *E. COLI* IN BULK MILK SOLD DIRECTLY FROM PRODUCER TO CONSUMER

Submitted to the *Journal of Food Protection*

5.1. ABSTRACT

Shigatoxin producing *E. coli* (STEC) in raw and pasteurised producer-distributor bulk milk (PDBM) has been recently reported in South Africa. Quantitative microbiological risk assessment and predictive modelling are important scientific tool which provides evidence-based and transparent estimation of the risk of foodborne illnesses. This study was envisaged to estimate the haemolytic uraemia syndrome (HUS) risk associated with consumption of STEC contaminated PDBM and estimate the resulting burden of illness that may be associated with consumption of PDBM in South Africa. Data was obtained from recently completed studies in South Africa taking into account prior collected prevalence data of STEC in raw and pasteurised PDBM, and survey information from producer-distributor (PD) outlets and households. Inputs for the models were complemented with data from published and unpublished literature. A probabilistic exposure model was developed with Monte Carlo simulation in Excel add-in software using @Risk software. Hazard characterisation was based on an exponential dose-response model to calculate the probability of illness from STEC in age groups below and above 5 years. The mean estimated STEC concentration was 0.12 colony forming units (CFU)/ml (95% CI: 0 – 1.2; $\sigma = 0.34$), for raw PDBM and 0.08 CFU /ml (95% CI: 0 – 1; $\sigma = 0.27$), for pasteurised PDBM. A higher risk of HUS cases per year was recorded in raw than pasteurised PDBM and also in age groups below 5 years. For every 100 000 PDBM portions consumed, the expected median number of HUS cases per-year were 52 and 3.2 for age groups under 5 years and above 5 years in raw PDBM. The median cases per-year attributable to pasteurised PDBM were 47 and 2.9 for age groups under 5 years and above 5 years, respectively. Sensitivity analysis revealed that serving volume and time taken to sell PDBM at PD outlets as factors with the greatest impact on probability of illness. Results from this study can be useful in formulating risk-based mitigation strategies and policies. Additionally, the

models developed in this study are an example of risk assessments for milk produced and marketed from similar scenarios across the globe.

5.2. INTRODUCTION

Over the years, shigatoxin producing *Escherichia. coli* (STEC) have globally evolved from clinical novelty to primary food safety and public health concern (Khan et al., 2002). Long-term sequelae of STEC infections range from mild diarrhoea and intestinal discomfort to serious complications such as haemolytic-uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). STEC O157:H7 is still recognised as epidemiologically significant world-wide, however, in some geographical regions, non-O157 STEC (O26, O103, O111 and O145) are becoming prominent as important *E. coli* pathotypes (Khan et al., 2002; Delignette-Muller et al., 2008). Ntuli et al. (2017) documented emerging non-O157 STEC O2, O9, O20, O43, O64, O68, O83, O112, O155 and O157 in PDBM in South Africa (SA).

Documented milkborne disease outbreaks have been linked to consumption of both raw (CDC, 2007; Denny et al., 2008; Claeys et al., 2013) and pasteurised (Goh et al., 2002) bulk milk contaminated with shigatoxin producing *E. coli* (STEC), in particular O157. EFSA (2015) documented 27 foodborne outbreaks in Europe attributed to STEC in bulk milk. During the period 2007-2012, thirteen outbreaks associated with STEC in bulk milk were recorded in 26 states in the US (Mungai et al., 2015). Epidemiological statistics on STEC in food in Africa are imprecise and the studies are few, although a recent review linked several outbreaks of STEC to food in the region (Raji et al., 2006). In SA, there are no official data existing on the prevalence of STEC linked to contaminated food. However, studies have indicated prevalence of STEC isolated from humans and livestock faeces, water and food, ranging from 15% to 42.8% (Aijuka et al., 2014;

Iweriebor et al., 2015; Ndlovu et al., 2015; Ntuli et al., 2017). A survey on producer-distributor bulk milk (PDBM) in SA revealed high levels of *E. coli* (Ntuli et al., 2016; 2017), above the stipulated limit (SA, 2001 Act (54), (1972)). Ntuli et al. (2016; 2017) also reported a diversity of STEC seropathotypes, (with different shigatoxin virulence factors, multi drug resistance and extended-spectrum β-lactamases (ESBLs) producing capacity in the PDBM. Other research studies carried out on *E. coli* isolated from bulk milk in SA by Caine et al. (2014) and Msolo (2016), documented diarrheagenic *E. coli* belonging to enterohaemorrhagic *E. coli* (EHEC).

Producer-distributor bulk milk in SA is typically (i) raw milk for human consumption, (ii) raw milk received with the intention to process and sell as pasteurised milk, and (iii) pasteurised milk received to be sold, that has been pasteurised elsewhere at an approved facility. This milk constitute 2% of total milk produced and sold in SA. Only state certified producer-distributors (PDs) are permitted to sell raw milk directly to consumers in SA (SA, 2001 Act (54), (1972), however unregistered/anuthorised producers also find their way in the PDBM value chain. The sale of PDBM directly to consumers is a common practice in SA and around the world. In SA, no attempts have been made to quantify the risk posed to human health by pathogens in milk.

To gain an insight on the accurate estimates of the actual risk posed by consumption of PDBM contaminated by pathogenic *E. coli*, a quantitative microbial risk assessment modeling is one of the methods to evaluate food health risks and control (CAC, 1998; FAO/WHO, 2003). Several risk assessment studies have been conducted in US, Europe and Africa in an attempt to quantify disease cases as a result of milk borne pathogens, (Grace et al., 2008; Clough et al., 2009; Giacometti et al., 2015; Giacometti et al., 2016).

Owing to the lack of epidemiological data, the burden of pathogenic *E. coli* linked to consumption of PDBM in SA has not been assessed. This study conducted a quantitative risk assessment of STEC in PDBM under the current production and marketing conditions in SA. The study was envisaged to estimate the HUS risk associated with consumption of STEC contaminated PDBM. This will enable assessment of factors that would have the greatest impact on public health and safety along the PDBM supply chain as well as formulating hypothetical mitigation strategies. Furthermore, this risk analysis facilitated the identification of data scarcity, which needs to be addressed for future quantitative risk assessments on PDBM. The models developed in this study are an example for other risk assessments in milk produced and marketed from similar scenarios across the globe.

5.3. MATERIALS AND METHODS

5.3.1. Hazard identification

Recent reports on PDBM in SA indicated prevalence of STEC O157 and non-O157 ranging from 10 – 54% (Caine et al., 2014; Msolo, 2016; Ntuli et al., 2016; Ntuli et al., 2017). Lately, milkborne disease outbreaks were incriminated to consumption of raw milk contaminated with STEC in the EU and US, especially raw milk sold directly from producer to the public (CDC, 2007; Denny et al., 2008; EFSA, 2015). A review by Claeys et al. (2013) reported 13 *E. coli* outbreaks in Europe and 28 worldwide associated with consumption of raw milk, between 1970 and 2010. The same authors documented an increased incidence consisting of 27 STEC outbreaks in Europe between 2007 and 2012 as a result of raw milk consumption. However, few outbreaks were also reported for pasteurised milk during that period (Clough et al., 2009). Farrokh et al. (2013) documented STEC outbreaks from 1986 - 2010 that have been linked to milk and dairy products (such as

cheese, yoghurt and ice cream) in Europe, US and Canada. Most of these outbreaks, reviewed by Farrokh et al. (2013), were associated with STEC O157, although other serotypes or serogroups, including O22:H8, O110:H$^-$, O80:H$^-$, and O145 have also been identified as causative agents. *E. coli* can grow at a temperature range of 7 to 46 °C with an optimum of 37 °C, however, studies have shown that depending on the food matrix, the organisms can resist pasteurisation temperatures of up to 72 °C (Mercer et al., 2015). Faecal-contaminated foods including, raw vegetables, under-cooked beef burgers, milk and milk products, are the most common vehicles for transmission of STEC from animals to humans.

5.3.2. Hazard characterisation

Virulence properties, mechanisms of pathogenicity, clinical symptoms and distinct serogroups are used to distinguish different *E. coli* strains that cause diarrheal diseases. Albeit, effects of STEC being dependent on host susceptibility (immune status and immunity imparted by previous exposure) and dose ingested, the most vulnerable members are children under 5 years, the elderly and immune-compromised individuals. However, some STEC strains (O104 and O157 serotypes) have been proven to cause severe illnesses even in healthy adults (Mellmann et al., 2011). STEC pathotypes exhibit different clinical syndromes with distinctive pathological and epidemiological characteristics of disease (Robins-Browne, 2004). The young, immunocompromised and elderly are at particular risk for the progression of the disease to more severe sequelae yet in healthy adults infection may be restricted to watery diarrhoea or may even be sub-clinical (Clark, 2009). Long-term sequelae of STEC infections range from mild diarrhoea and intestinal discomfort to serious complications such as HUS and TTP. Studies have shown that a few STEC cells are necessary to cause illness. Ingestion of 5 – 50 cells of STEC can cause fatal illness to humans (Delignette-Muller et al., 2008; Nguyen and Sperandio, 2012; Dean et al., 2013).

5.3.3. Exposure assessment

5.3.3.1. Field survey

A survey was conducted in urban and peri-urban parts of Pretoria in SA (one of the PDBM sampling areas) with the aim of getting an insight on the typical flow of PDBM from outlets to consumer and PDBM consumption patterns. A questionnaire was developed to capture the following information: (i) average volumes of PDBM produced or received at outlets per-day (ii) PDBM handling practices and storage conditions at outlets, (iii) average volumes of PDBM sold per-day, (iv) PDBM handling practices during transportation to home, (v) consumer handling practices and storage conditions, and also consumption patterns. A total of 15 PDBM outlets and 80 consumers were interviewed and the information was used as input for the models.

5.3.3.2. Overview of PDBM pathway to consumer and exposure model

Consumers of PDBM obtain their milk from different sources either as raw or pasteurised. Stages prior PD outlets were not included in this model. The model was developed starting from PD outlets to household level for PDBM which was sold either as raw or pasteurised. The conceptual model upon which the mathematical model was based to estimate the exposure of STEC to consumers is depicted in Figure 5.1. A "modular process risk" framework (Nauta, 2002) was adopted to simulate the scenario which the milk undergoes from the PD outlets to consumption. The same scenario was used for either raw or pasteurised PDBM, however, what differed was the STEC prevalence and concentration in raw and pasteurised PDBM at the time of sell at PD outlets (Ntuli et al., 2016). Consumers either brought containers which were filled directly from the bulk tank or they bought small plastic containers (1 to 5 L) prefilled with bulk milk at the outlets. Changes in prevalence and concentration of STEC in PDBM from outlets to consumption after

storage at home were modeled. At each step, basic microbial and milk handling processes, such as growth and partitioning were identified and applied. The model was divided into the following steps: (i) PD storage, (ii) transport time and temperature from PD to home and consumer handling, and (iii) consumption habits at home and exposure to STEC. Each step, in sequence, produced one or more output distributions that served either as inputs to the next step or as final outputs of the estimation of the probability and concentration in a single serving at consumption. The model was developed from input data derived from the field survey (section 5.3.3.1), a completed study on PDBM by Ntuli et al. (2016), other published literature and expert opinion whenever possible. Input parameter variables, their description and associated equations or distributions, for PDBM production model are presented in Table 5.1. The same model was used for both raw and pasteurised PDBM.

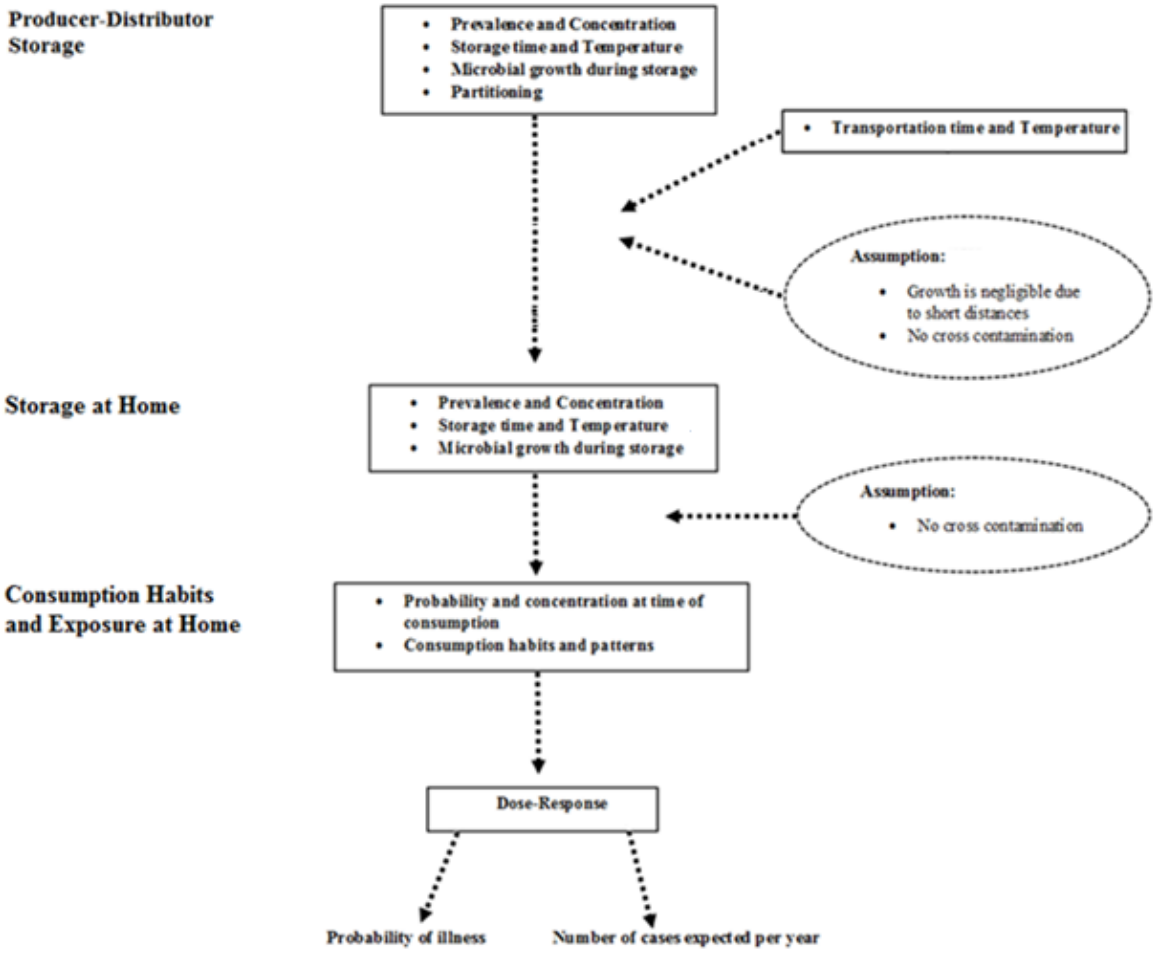

Figure 5.1: Schematic overview of the quantitative risk assessment model for shigatoxin producing *E. coli* in producer-distributor bulk milk.

5.3.3.3. Estimation of STEC concentration in PDBM

Data on the prevalence of STEC in raw and pasteurised PDBM samples reported in previous studies by Ntuli et al. (2016; 2017) were considered in the estimation of STEC concentration. The data reported positive for STEC in 17 (n=154) and 8 (n=104) raw and pasteurised PDBM samples, respectively. A direct plate count method using 3M petrifilm plates and molecular techniques was adopted by the authors, CFU/ml of STEC in the positive samples ranged from 1 to 3.

Poisson distribution was used to calculate the mean concentration of STEC in both raw and pasteurised PDBM (Sanaa et al., 2004). To test that STEC in PDBM follow a Poisson distribution, a chi-square goodness of fit test was carried out.

5.3.3.4. Producer-distributor storage

The distribution of PDBM volumes produced or received per day at outlets were computed from a study by Caine et al. (2014) and from the survey (Table 5.1). Milk is stored as either raw or pasteurised and the volumes of PDBM was incooperated in the model as a distribution representing variability in milk volumes at each outlet. As a result of limited data from the survey, uniform distributions were used to simulate variability in storage temperatures and time taken to sell all the milk from one batch and this was used in the growth model (Table 5.1). Cross contamination at PD storage is uncertain due to lack of data or published data, therefore, no cross contamination during modeling was assumed. Maximum population density (MPD) and maximum growth rate (μmax) of STEC at 9.5 ºC in milk used in the growth model was obtained from a study by Kauppi et al. (1996). Concentration after growth occurred during storage was computed taking into account, MPD, μmax, time all the milk is sold and the initial concentration (Table 5.1). Prevalence does not change since it is not affected by growth.

Consumers buy PDBM in plastic containers or the milk is packaged into retail units (0.5 to 5 L) at PD outlets. The model used to calculate STEC prevalence and concentration in smaller units assumed that STEC is randomly distributed in the milk. The new prevalence is the probability that at least one STEC cell is present in the new smaller units, given that the bulk milk where it is drawn was previously contaminated with STEC, was considered equal to the fraction of bulk milk that the small unit represents. Therefore, STEC prevalence in the containers is adjusted by the

probability that one or more STEC cells will end up in a random smaller unit. Concentration of STEC after packaging into smaller volumes was calculated by binomial sampling of the number of STEC cells that are in the small units. The new STEC concentration in randomly generated as, contamination count divided by the small unit volume (Table 5.1) (Njage and Buys, 2016). A uniform distribution was used to model the distribution of milk volumes sold per day and this was used to calculate number of servings consumed per day.

5.3.3.5. Transport from PD to home and consumer handling

Based on the survey, the assumption was that there was negligible or no STEC growth during transportation of PDBM, given short distances and time from outlets to home, even though the milk was transported at abused temperatures (Table 5.1). On the basis of interview answers, milk was consumed up to 5 days especially when refrigerated. Hudson and Hartwell (2002) and Marklinder et al. (2004) noted that there is variability in refrigeration temperatures in homes that can allow *E. coli* growth in food. The authors observed that 39% of households refrigerated their food at 6 – 7 °C, 4% at 7 – 8 °C, 4% at 9 – 10 °C and 1% at 12 ºC. A cumulative distribution of the different refrigeration temperatures from different proportions of consumer was used as an input for the growth model at home storage. Growth was further computed taking into account STEC concentration after packaging into smaller units, maximum growth rate, distributions representing variability of time taken to transport the milk from PD to home and storage at home, and MPD of STEC in milk at refrigeration temperatures (Kauppi et al., 1996). Maximum growth rate (μmax) of STEC at refrigeration temperatures was derived from a cumulative distribution of the different μmax values at 6-7, 7 – 8, 9 – 10, 12 ºC and proportions of consumers storing milk under the respective different refrigeration temperatures (Hudson and Hartwell, 2002; Marklinder

et al., 2004). Prevalence after growth during storage at home in refrigerators was considered unaffected (Table 5.1).

5.3.3.6. Consumption habits at home and exposure to STEC

According to the interviews carried out in the survey, 67% of the consumers boiled PDBM before consumption. Giacometti et al. (2012b) observed that boiling milk completely eliminates viable *E. coli* cells. However, the remaining 33% used methods such as microwave, mixing with hot tea or porridge, which were consider as inadequate/insufficient heat treatment of milk. Log reduction for the insufficient heat treatment of milk was modeled using a triangular distribution (Giacometti et al., 2012a) (Table 5.1). In the current study, frequency of PDBM consumption for children under 5 years was higher than the population above 5 years (Table 5.2). According to survey estimations, the population under 5 years consumed 124 984 915 portions of milk per-year and the age group above 5 years consumes 43 473 014 portions per-year. Consumer habits was used in the final exposure model. The distribution of PDBM serving size was characterised by values from the survey (Table 5.1). Final exposure (concentration) of STEC per serving was calculated as an output using the model in Table 5.1.

5.3.4. Dose response

The dose response model of STEC in food used by Delignette-Muller et al. (2008) was adopted in this study. The authors directly modeled the probability of HUS as a function of ingested dose. Probability of illness from STEC infections is dependent on age and other factors as reported by Nauta et al. (2001). In the current study, two dose-response models were used for two age groups, 0 to 5 and > 5 years (Table 5.2). Children under 5 years are more susceptible to STEC as documented from an epidemiological study (Loirat et al., 2008). The values for *r* used in the model

were estimated by Delignette-Muller et al. (2008) for each age group (Table 5.2). Probability of HUS per-serving was computed by combining the dose estimate and contamination prevalence (Ross et al., 2009). Number of cases per year was calculated by multiplying the probability of HUS per-serving and the number of serving per year for each target age group (Table 5.2).

5.3.5. Simulation and analysis

Stochastic modelling of the exposure with STEC for all scenarios were implemented with the Monte Carlo simulation technique by using the risk analysis software @Risk 7.5 (Palisade Corporation, Ithaca, USA). All models were simulated for 100 000 iteration as carried out by Latorre et al. (2011) and Giacometti et al. (2012a). The outputs of the model was the median risk of HUS per-serving in each class of consumers' ages. Sensitivity analysis for each scenario was performed to identify important parameters from their corresponding distributions. Spearman's correlation coefficient was used to estimate the impact of PDBM value chain practices on the variability in exposure with STEC in PDBM per-year. Possible PDBM handling scenarios were introduced to test the associated effects in reducing exposure per-serving to STEC to consumers who insufficiently heat the milk. The scenarios include: storage and handling of PDBM at 4 °C throughout the whole chain, time taken to sell the milk at PD outlets, time taken to consume all the milk at home and a combination of, some of the, PDBM handling practices. Storage and handling of PDBM at 4 °C throughout the whole chain was broken down into points where this storage effect was modeled (PD outlets, transportation to home and home refrigeration). In the present study, 5, 6 and 7 h were selected as time taken to sell the milk at PD outlets. This is the possible and realistic time PDs can acquire milk and sell within the same day. Maximum recommended time for raw milk storage at house hold is 3 days (Giacometti et al., 2012a), therefore, half, one and two days was used as possible and realistic time taken to consume all the

milk at home. A combination of scenarios (storage at 4 °C throughout the whole chain + time taken to sell the milk (5 h) + time taken to consume the milk (half a day)) was also evaluated.

Table 5.1: Input parameters for exposure model of STEC in raw and pasteurised producer-distributor bulk milk: Description, equations or distribution, values and units of the input parameters and data sources.

Steps	Parameter	Description	Distribution/ Equation/values	Units	Data Source/reference
Producer-distributor storage	IP_{PDS}	Initial prevalence of STEC positive PDBM	11 raw milk 8 pasteurised milk	% %	(Ntuli et al., 2016)
	IC_{PDS}	Initial concentration of STEC in PDBM samples	0.12 raw milk 0.08 pasteurised milk	CFU/ml	
	V_{outlet}	Volume produced or received at outlets.	RiskPert(500000, 1000000,5000000)	ml	(Caine et al., 2014) Survey
	$Temp_{PDS}$	PDBM storage temperature	RiskUniform(8,11)	°C	This study
	$Time_{PDS}$	Time taken to sell PDBM	RiskUniform(24,48)	h	This study
	$Milk_{sale/day}$	Average PDBM sale per-day	RiskUniform(500000, 5000000)	ml	This study
	G	Growth of STEC during PDBM storage	$Time_{PDS}$ in hr x µmax Where: µmax = 0.036	log CFU log CFU/h	(Kauppi et al., 1996)
	$Conc_{PDS}$	Concentration of STEC after growth occurs during storage of PDBM	$IF(IC_{PDS} \times 10^{G})>MPD,MPD,(IC_{PDS} \times 10^{G})$ Where: MPD = 31622777	CFU/ml CFU/ml	

	$Size_{PDS}$	Volume of milk sold to consumer and size of containers	RiskPert(500,1000,5000)	ml	This study
	$Prev_{consumer}$	New PDBM sample prevalence with STEC after packaging into smaller units	IP_{PDS} x $Psmall_{consu}$ Where: $Psmall_{consu} = (1-(1-(Size_{PDS}/V_{outlet}))^{Nconsu}$ $N_{consu} = round(Conc_{PDS} \times V_{outlet})$		
	$C_{consumer}$	New PDBM STEC concentration after packaging into smaller units	RiskBinomial($N_{consu,}$ $Size_{PDS}$ /V_{outlet}) / $Size_{PDS}$	CFU/ml	
Transport time and temperature from producer-distributor to home and Consumer handling	$Trans_{Temp}$	Transportation temperature	RiskPert(14.3, 26, 38)	°C	This study
	$Trans_{Time}$	Transportation time	RiskUniform(0.5,3)	h	This study
	$Time_{shelflife}$	Time until all PDBM is consumed at home	RiskPert(1,2,5)	days	This study
	$Temp_{fridge}$	Temperature refrigeration at home	RiskCumulD(5.5,12,{5.5,7.5,9.5,12}, {0.39,0.04,0.04,0.01})	°C	(Hudson and Hartwell, 2002; Marklinder et al., 2004)
	G1	Growth of STEC in PDBM during refrigeration storage at home	$Time_{shelflife}$ in hr x µmax Where: µmax = RiskCumulD(0.019,0.041,{0.019,0.028,0.036,0.041}, {0.39,0.04,0.04,0.01})	log CFU log CFU/h	

	C_{HST}	Concentration STEC in PDBM after growth occurs during refrigeration storage at home	IF($C_{consumer}$ x 10^{G1})>MPD,MPD,($C_{consumer}$x 10^{G1})		
Consumption habits at home and exposure to STEC	$Serving_{size}$	Milk serving size at consumer level	RiskPert(150,500,1000)	ml	This study
	$Time_{GT-EX}$	Generation time of STEC under refrigeration conditions	RiskTriangle(34.2, 45.1, 56)	h	(Giacometti et al., 2012b)
	$Time_{shelflife}$	Time milk is stored and consumed at home	RiskPert(1,2,5)	h	This study
	B_{EX}	Consumer habits (milk boiling before consumption at home)	RiskBernoulli(0.67) x D_{EX}		
	D_{EX}	Log reduction of those who insufficient heat the milk at consumer level	RiskTriangle(2,4,6)	log CFU/ml	(Giacometti et al., 2012a)
	$Dose_{per-serving}$	Dose of STEC per serving	10^(log[10^{CHST} x 2^(Timeshelflife / TimeGT-EX)] - B_{EX}) x $Serving_{size}$	CFU	(Giacometti et al., 2012a)

Table 5.2: Dose response assessment for STEC in raw and pasteurised producer-distributor bulk milk: Description, equations or distribution, values and units of the input parameters and data sources.

Parameter	Description	Distribution/ Equation/values	Units	Data Source/reference
P_{HUS}	Probability of haemolytic uremic syndrome	$P_{HUS} = 1 - (1 - r)^D$ Where: *r* is the dose response parameter per organism: r = 1.28×10^{-3} (0 to 5 years) r = 2.4×10^{-4} (> 5 years) *D* is dose per-serving ($Dose_{per\text{-}serving}$)	CFU	(Delignette-Muller et al., 2008)
$P_{Serving}$	Probability of illness per-serving	$P_{Serving} = P_{HUS}$		(Latorre et al., 2011)
N_{case}	Number of haemolytic uremic syndrome cases per year	$P_{Serving}$ x Number of servings per year Where: Number of servings per year = frequency of consumption per month for each age group x portions consumer with insufficient heat treatment.		(Latorre et al., 2011)
		Frequency of consumption per month for each age group: (0 to 5) = RiskUniform(16,30) >5 years = RiskPert(0,8,16)		This study
		Portions with insufficient heat treatment = 0.33 x portions consumed per year		This study

Where: 0.33 is proportion of consumers who insufficiently heat the milk

Portions consumed per year = ((Milk$_{sale/day}$ x 178)/Size$_{PDS}$) x 365 days (MPO, 2016)

Where: 178 is number of PDs in SA

5.4. RESULTS

5.4.1. Concentration of STEC in raw and pasteurised PDBM

From the prevalence data on STEC positive samples, the estimated number of STEC in raw (0.12 CFU/ml) and pasteurised (0.08 CFU/ml) PDBM was done by fitting a Poisson distribution. A Chi-square goodness of fit test was used to test the fit of the Poisson distribution. Pearson χ^2 at 1.79, degrees of freedom was 2 and *p*-value based on χ^2 distribution was 0.20 for raw PDBM. For pasteurised PDBM, Pearson χ^2 at 1.90 degrees of freedom was 1 and *p*-value based on χ^2 distribution was 0.11. Therefore, the Poisson distribution adequately predicted the estimated number of STEC in both PDBM types. The model gave a mean STEC estimate concentration of 0.12 CFU/ml (95% CI: 0 – 1.2; σ = 0.34 CFU/ml), for raw PDBM and 0.08 CFU/ml (95% CI: 0 – 1; σ = 0.27), for pasteurised PDBM.

5.4.2. Exposure assessment

The estimated levels of STEC per-serving, after boiling the milk, in both raw and pasteurised PDBM are depicted in Table 5.3. The quantity of STEC that a consumer was exposed to in a single serving of milk was a function of the initial concentration of STEC in PDBM at PD outlets, and the subsequent effects of handling and storage along the milk chain. STEC levels increased during storage at PD outlets and home refrigeration, reaching microbial loads of 42 (95% CI: 15 – 569) CFU/per-serving in raw and 28 (95% CI: 10 – 385) CFU/per-serving in pasteurised PDBM, prior heat treatment. Considering the 33% of consumers who insufficiently heat the milk before consumption, the STEC concentration per-serving ranged between 3.2×10^{-4} and 7.3×10^{3} CFU/per-serving for raw PDBM and 2.95×10^{-4} to 6.42×10^{3} CFU/per-serving for pasteurised

PDBM (Table 5.3). The median STEC concentration per-serving was 0.42 CFU/per-serving and 0.37 CFU/per-serving in raw and pasteurised PDBM, respectively, for the 33% of consumers who insufficiently heat the milk before consumption. The model predicted prevalence of PDBM contaminated with STEC to be 11% and 8% for both raw and pasteurised PDBM at the time of consumption.

5.4.3. Risk characterisation

To assess the risk posed to consumers from consuming STEC contaminated PDBM, the exposure assessment model was used and each iteration predicted a probability of illness and consequently the number of HUS cases per-year (Table 5.4). In simulations where all consumers boil milk before consumption, no risk was calculated for both raw and pasteurised PDBM. The model estimated higher probability of illness per-serving for consumers of raw than pasteurised PDBM, when considering consumers (both age groups) who insufficiently heat the milk before consumption. The highest median probability of illness per-serving was noted in children under 5 years for raw PDBM (5.4×10^{-4}), while consumers of pasteurised PDBM who are above 5 years of age recorded the least median probability (9.0×10^{-5}) of illness pre-serving (Table 5.4). Number of HUS cases per-year were also higher for children under 5 years of age than consumers above 5 years of raw and pasteurised PDBM. For every 100 000 portions of PDBM, the highest recorded median number of HUS cases per-year (52) was observed in consumers below 5 years who consume raw PDBM (Table 5.4). The median cases per-year attributable to pasteurised PDBM were 47 and 2.9 for age groups under 5 years and above 5 years, respectively, for every 100 000 pasteurised PDBM portions consumed.

Table 5.3: Estimation of shigatoxin producing *E. coli* concentration per-serving in raw and pasteurised producer-distributor bulk milk.

Parameter / percentile	Estimated level of STEC (CFU/per-serving)	
	Raw PDBM	**Pasteurised PDBM**
Minimum	3.2×10^{-4}	2.95×10^{-4}
Mean	4.1×10^{2}	3.66×10^{2}
Maximum	7.3×10^{3}	6.42×10^{3}
5^{th}	5.84×10^{-3}	5.12×10^{-3}
50^{th}	0.42	0.37
95^{th}	1.88×10^{3}	1.69×10^{3}

Table 5.4: Probability of illness per-serving and number of haemolytic-uremic syndrome cases per-year with consumption of raw and pasteurised producer-distributor bulk milk.

Milk category	Population	Probability of illness per-serving Median (5^{th}, 95^{th}) percentiles	Number of cases per-year Median (5^{th}, 95^{th}) percentiles
Raw PDBM	Under 5 years	5.4×10^{-4} (7.5×10^{-6}, 0.91)	52 (0.68, 1.3×10^{5})
	Above 5 year	1.0×10^{-4} (1.4×10^{-6}, 0.37)	3.2 (0.04, 1.5×10^{4})
Pasteurised PDBM	Under 5 years	4.8×10^{-4} (6.7×10^{-6}, 0.82)	47 (0.60, 1.2×10^{5})
	Above 5 year	9.0×10^{-5} (1.3×10^{-6}, 0.33)	2.9 (0.03, 1.3×10^{4})

*Values are the median, 5^{th} and 95^{th} percentile obtained after 100 000 iteration, using @ risk 7.5 in both raw and pasteurised PDBM
*The values for number of cases were calculated for every 100 000 portions of PDBM consumed.

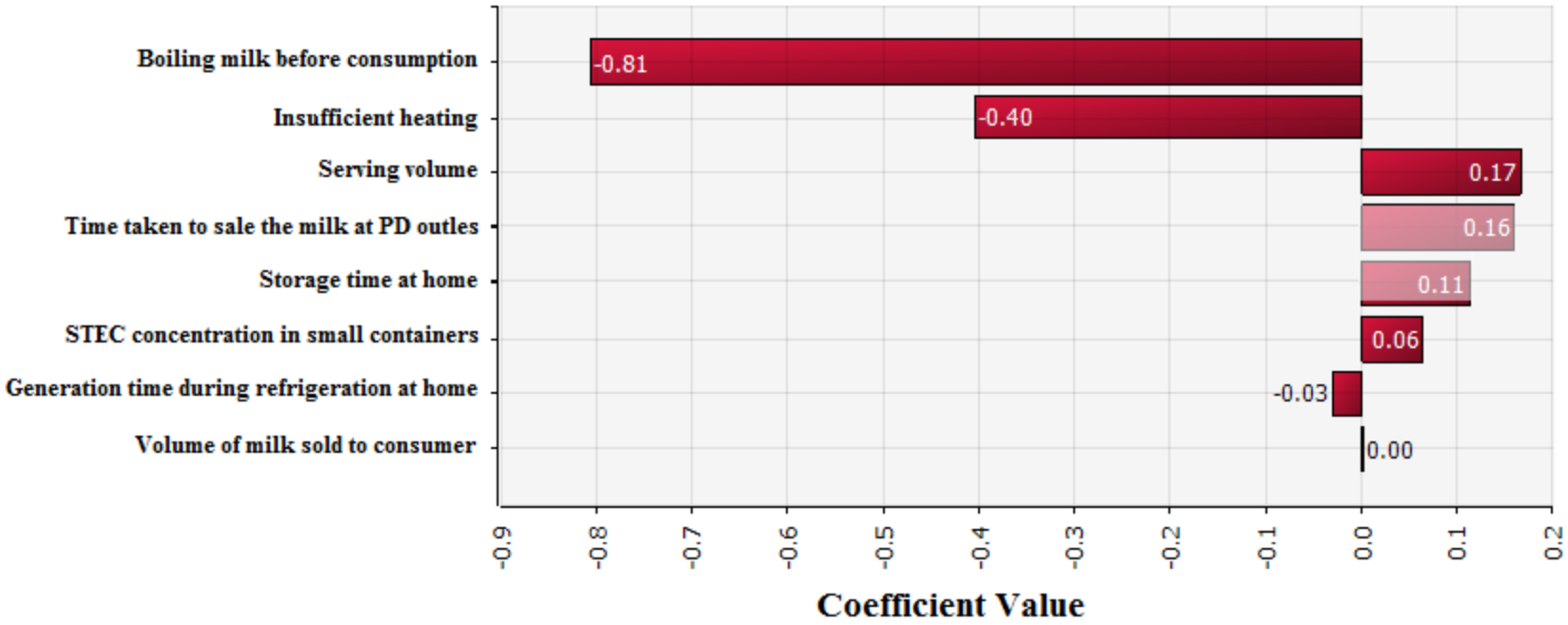

Figure 5.2: Sensitivity analysis between estimated probability of illness after one serving of producer-distributor bulk milk and important predictive factors along the value chain.

5.4.4. Effect of model parameters on the risk of HUS

In the current study, sensitivity analysis on the models indicated that serving volumes (Spearman's correlation coefficient (ρ) = 0.17) had the greatest effect on the probability of HUS and the annual number of cases (Figure 5.2). Time taken to sell the milk at PD outlets (ρ = 0.16) and PDBM storage time at home (ρ = 0.11) were also important factors that influenced probability of HUS and the annual number of cases. After packaging bulk milk into small containers for the consumer, the new modeled concentration of STEC (ρ = 0.06) also affected probability of illness. Heat treatment of PDBM before consumption, greatly reduced the level of STEC and the subsequent risk of HUS in both raw and pasteurised PDBM (Figure 5.2). Generation time of STEC during refrigeration at home, which is achieved by proper refrigeration, also reduced the risk of HUS (ρ = - 0.03)

5.4.5. Possible PDBM handling scenarios

Storing PDBM at 4 °C throughout the whole chain revealed that it was most effective when applied at PD outlets in both raw (23.1% reduction of STEC concentration) and pasteurised (19.6% reduction of STEC concentration) PDBM. The study observed that, as time to sell PDBM and time to consume the milk after arriving at home was reduced, the concentration of STEC per-serving also reduced significantly (Table 5.5). Reducing time taken to consume all the milk after arriving to half a day, was the most effective single handling practice, with 55.8 and 57.1% reduction in STEC concentration per-serving in raw and pasteurised PDBM, respectively. Considering time taken to sell the milk at PD outlets, the highest reduction in consumer exposure to STEC was observed when milk is received and sold to consumers within 5 h per-batch (54.2 and 56.0% reduction of STEC concentration in both raw and pasteurised PDBM respectively). Combining

possible handling scenarios (storage at 4 °C throughout the whole chain + time taken to sell the milk (5 h) + time taken to consume the milk (half a day)) was the most effective practice in reducing consumer exposure to STEC for both raw (83.2% reduction of STEC concentration) and pasteurised (88.5% reduction of STEC concentration) PDBM.

Table 5.5: Possible handling scenarios and their associated effects in reducing exposure per-serving to shigatoxin producing *E. coli* to consumers who do not boil producer-distributor bulk milk.

Handling procedures	**Reduction in concentration of STEC per-serving (%)**	
	Raw PDBM	Pasteurised PDBM
[a]Storage and handling at 4 °C:		
PD outlets	23.1	19.6
Transportation home	8.0	9.7
Home refrigeration	13.3	11.9
[b]Time taken to sell the milk:		
5 h	54.2	56.0
6 h	51.8	54.3
7 h	44.0	45.1
[c]Time taken to consume all the milk at home:		
½ a day	55.8	57.1
1 day	43.5	46.4
2days	34.9	37.2
a (4°C)+b (5 h)+c (½ a day)	83.2	88.5

5.5. DISCUSSION

In this present study, a stochastic quantitative microbial risk assessment was carried out, from PD outlets to consumption, of HUS associated with the consumption of STEC contaminated PDBM based on results from the study by Ntuli et al. (2016; 2017) and also from a survey carried out in

one of the sampling areas in SA. This provided an estimate of the nationwide PDBM scenario of HUS cases that may be linked to the consumption of STEC contaminated PDBM.

STEC concentration was lower in pasteurised (0.08 CFU/ml) than raw (0.12 CFU/ml) PDBM. Under ideal conditions, no STEC cell survives pasteurisation temperatures (Goh et al., 2002). However, a previous study by Ntuli et al. (2016) recorded presence of alkaline phosphatase in 21% (n=104) of PDBM samples. The authors could not establish the possible source and pathway of STEC in pasteurised PDBM, although this was explained as either inadequate pasteurisation process or contamination/cross-contamination of a batch of PDBM after a successful pasteurisation (Ntuli et al., 2016). Using a modeling approach, Clough et al. (2009) highlighted that STEC contamination in milk occurs either from inadequate pasteurisation or post-pasteurisation contamination. In their study, they reported that, although inadequate pasteurisation may result in survival of STEC, subsequent dilution effects lowers the health risk associated with STEC to very low levels in packed milk.

The developed model in this study also assessed the risk introduced during consumer handling. This consist of steps after consumer purchase and the subsequent handling at household level. At these stages, PDBM is no longer controlled by professionals (Nauta and Christensen, 2011; Crotta et al., 2016). Temperature and time of milk handling and storage was treated as independent parameters. This may have overestimated the risk of HUS since an implicit assumption, underlying the model, is that all the milk will be consumed whatever the time-temperature combinations. Practically some milk can end up not being consumed due to spoilage at certain time-temperature combinations thereby reducing the risk. A study by Crotta et al. (2016) developed a model which captured the dependencies between time and temperature to express the likelihood for milk serving to be actually consumed for any computed storage time-temperature combination and extent to

which the dependency would affect the output. However, the scenarios they used may not apply for the PDBM scenario in SA.

Exposure concentration of STEC in PDBM per-serving was dependent on the estimated concentration of STEC at PD outlets. Raw PDBM had higher concentrations of STEC per-severing than pasteurised PDBM for both age groups. Based on the survey that was conducted, the frequency of consumption was 2.8 times higher in children under 5 years than the population above 5 years (data not shown). Albeit, children 0 – 5 years may consume smaller portions/volumes of milk than age groups above 5, the probability of illness and number of HUS cases was higher in children under 5 based on frequency of consumption. Furthermore, Signorini and Tarabla (2009) reported that children under 5 have an increased probability, due to low infective dose, of severe disease outcomes such as HUS and death following STEC infection. Findings from this study was in contrary to observations by Delignette-Muller et al. (2008) who conducted a risk assessment for STEC in frozen ground beef patties consumed in France. The authors noted that risk of HUS was high in age groups above 5 years because of portion sizes of food consumed. The probability of illness for both the age groups consuming PDBM were extremely small (far less than 1) but this is difficult to validate given the uncertainty which underlie in the number of PDBM milk consumers. There are no official reports on HUS cases in SA to benchmark this model outputs. However, in Italy, STEC risk assessment for milk reported similar cases of HUS as reported by the Health Ministry (Giacometti et al., 2012a; Giacometti et al., 2016). Latorre et al. (2011) in the US conducted a risk assessment of listeriosis due to consumption of raw milk and also reported a number of listeriosis cases which were in line with reports from the CDC. In the current study, for every 100 000 PDBM portions per-year, the expected number of HUS cases per-year were 52 and 3.2 for age groups under 5 years and above 5 years, respectively, in raw PDBM and the expected

number of HUS cases per-year in pasteurised PDBM were 47 and 2.9 for age groups under 5 years and above 5 years, respectively. Results from this study differ considerably with those of Grace et al. (2008), who reported a higher estimate of 2.40 to 2.83 cases of STEC infections per 10,000 servings portions of raw milk. Giacometti et al. (2012a) also predicted that cases of HUS per-year for 5.25 million portions of milk were 0.09 and 0.5 for children under 5 years and age group above 5 years per-year, respectively. Latorre et al. (2011) reported that disparities in model output can be as a result of the risk model and the data used in each model, for example temperature distributions, time distributions as well as prevalence of the pathogen in context. Median probability of illness per-serving for STEC in PDBM varied from 9.0×10^{-5} to 5.4×10^{-4} for all age groups. In Europe the reported median probability of illness per-serving for STEC in milk ranged from 9.36×10^{-11} to 2.56×10^{-3} (EFSA, 2015). The median probability of illness per-serving for STEC in PDBM in this study was very low and within the range of the findings from studies conducted in Europe. Nevertheless, cases of HUS and hospitalisation due to STEC infections have been reported in Europe despite the low probability of illness (EFSA, 2015).

In the current study, the risk of infection and the subsequent development of HUS was most influenced by serving volumes followed by time taken to sell the milk at PD outlets. These factors were the most important in increasing the risk of HUS in both age groups who consume either raw or pasteurised PDBM. Latorre et al. (2011) also reported serving volume as a parameter with great influence in the risk of listeriosis in raw milk (correlation coefficient varied from 0.19 to 0.30 for all the scenarios they studied). In the current study, the higher number of HUS cases estimated by the model for children under 5 years, although they consume smaller milk volumes, could be attributed to frequency of consumption (higher in the under 5) and infectious dose (lower in the under 5). In this study, the STEC exposure per-serving was very high for both raw and pasteurised

PDBM compared to results in a report by the FDA (2003). This could explain why sensitivity analysis picked serving volume as the most important parameter. Partitioning of milk into smaller containers also had an influence, although very low ($\rho = 0.06$), in the risk and probability of illness in this current study. During partitioning, aerial contamination from the surrounding environment can take place (Ledenbach and Marshall, 2010). Although this is insignificant under normal production conditions the concentration of microorganisms can further increase due to their growth. Therefore, extreme caution needs to be taken during partitioning. Heat treatment of milk greatly reduced the risk of HUS associated with consumption of STEC contaminated milk. Using a linear regression model, Giacometti et al. (2016) noted that the number of predicted HUS cases is directly influenced by the probability of heat treatment of milk before consumption and again that consumer behaviour is a variable and operational reference point useful to obtain appropriate mitigation measures. Grace et al. (2008), Giacometti et al. (2012a) and Clough et al. (2009) reported a zero risk of acquiring HUS in consumers who boil milk before consumption. *E. coli* is destroyed by temperatures above 63 °C in fluid milk (D'Aoust et al., 1988). Pasteurisation of milk effectively eliminates STEC and other common milk borne pathogens (*L. monocytogenes, Campylobacter* and *Salmonella*) that could cause severe disease, without causing significant change to nutritional properties in milk (Angulo et al., 2009).

A simulated scenario in this study where milk was stored at 4 °C throughout the whole PDBM chain, clearly indicated a reduction of HUS risk to consumers by more than 50%. The PDBM food chain should enforce handling, transportation and storage between 0 and 4 °C. These temperatures have been known to prevent microbial growth and subsequent risk of high pathogen level at consumption (Signorini and Tarabla, 2009). In their risk assessment, Giacometti et al. (2012a) observed that when farmers did not maintain correct temperatures throughout the supply chain

and also due to thermal abuse practices during home transportation and storage, the annual expected cases of HUS infections were higher. The same authors also reported that effective maintenance of the cold chain also reduces the risk of HUS associated with consumption of raw milk. The current study also noted that reduction of time taken to sell milk and consume all the milk at home, significantly reduced the risk of STEC in PDBM. Factors affecting risk of infection by pathogen in milk sold directly to the public include time taken to sell the milk per-day and time taken to consume all the milk at household level (Latorre et al., 2011; Giacometti et al., 2015) which is also in accordance with this current study. Latorre et al. (2011) reported that additional time in milk storage along the food chain increase growth of the pathogen and the subsequent exposure per-serving and risk of illnesses per-serving. This study therefore recommend consumption of milk within the shortest possible time just after purchasing, to reduce bacterial growth during inadequate refrigeration which has subsequent consequences of increasing the risk of infection. Studies have proven that *E. coli* cells can grow even at refrigeration temperatures (Kauppi et al., 1996). Combination of PDBM handling practices (storage at 4 °C throughout the whole chain + time taken to sell the milk (5 h) + time taken to consume the milk (half a day)) along the product chain had more impact in reducing the risk of infection and probability of illness. A study by Njage and Buys (2016) on quantitative assessment of human exposure to extended spectrum and AmpC ß-lactamases bearing *E. coli* in lettuce attributable to irrigation water and subsequent horizontal gene transfer, revealed that combination of mitigatory interventions, was effective in reducing the exposure with the *E. coli* by up to 99.4%. Most WHO guidelines recommend combination of different mitigatory measures in food value chain to increase food safety (Wilcock et al., 2004).

During the analysis carried out in this study, certain model inputs introduce uncertainties. A study by Caine et al. (2014) was identified apart from reports by Ntuli et al. (2016; 2017), which provides information regarding the prevalence of STEC in PDBM in SA or in the region. Furthermore, there is no information pertaining quantitative data of STEC and the inherent variability in this parameter in PDBM. In the current study, one of the main sources of uncertainty was the estimated concentration of STEC in both raw and pasteurised PDBM. Very few studies have quantified pathogen levels in bulk milk (Marshall et al., 2016). Trevisani et al. (2013) estimated the number of STEC O157 and O26 using the most-probable-number-PCR method. Most studies have used the Bayes' theorem techniques to quantify pathogen levels based on prevalence (qualitative) data in milk (Giacometti et al., 2012a; Giacometti et al., 2015). The level of STEC in PDBM was estimated based on the method of isolation and quantification that was used in previous study by Ntuli et al. (2016; 2017). One of the main disadvantage in the method was that, *E. coli* (STEC) cells are known to enter a dormancy state in the milk, i.e. they are still viable but non-cultrable (Dinu and Bach, 2011). Therefore, this may have underestimated the quantities of STEC in PDBM, although the cells may still be viable and retain pathogenicity. The most sensitive method for STEC isolation and quantification in food, including milk, is the immunomagnetic separation following selective enrichment, and subsequent spread-plating of the concentrated target cells onto STEC chromagar (Boer and Heuvelink, 2000). Obtaining quantitative data on STEC concentration in PDBM or milk produced and marketed in the same scenario, would enable a more realistic modelling at this PDBM value chain stage. In other studies, estimated concentrations of STEC in bulk milk ranged from -4.00 log CFU/ml to -3.5 log CFU/ml (Giacometti et al., 2012a; Perrin et al., 2015). However, these were much lower than what was estimated, in this current study, in raw and pasteurised PDBM despite the underestimations.

Storage temperatures at house hold refrigeration was modeled using data obtained from Europe and other western countries, and this might not be a representative of home refrigeration temperatures in SA. Another source of uncertainty and variability in the model was the lack of data available regarding (i) average volumes of PDBM produced or received at outlets per-day (ii) average volumes of PDBM sold per-day (iii) serving volumes (iv) percentage of consumers who boil milk before consumption (v) frequency of PDBM consumption (vi) the actual population (both children and adults) that consume PDBM in SA. Future risk assessment can also model this source of variability and uncertainty if appropriate data could be identified. Furthermore, sampling was done in a similar region where PDBM sample were collected in a previous study by Ntuli et al. (2016), as a representative of PDBM scenario in urban and pre-urban SA. This might have underrepresented the PDBM situation in SA since the socio-economic status in the country include a vast rural population who cannot access refrigeration and whose commute consists of walking for long distances. The milk may therefore be subject to more prevalence and levels of abuse temperatures between PD outlets and domestic levels. A triangular distribution was assigned to represent log reduction counts to represent insufficient heating (33% of the consumers) and this was adopted from Giacometti et al., (2012a). The authors reported this as a source of uncertainty in their model as the experimental data on the reduction of STEC counts achieved by insufficient boiling may not be reproduced in the home setup, thus, they assumed a triangular distribution. Regarding the set of data obtained in this study, it was not possible to estimate precisely the absolute risk of HUS in SA using the current model. The actual number of children and adults who consume PDBM was estimated from SA population statistics. Future risk assessment work is therefore recommended, that include other vulnerable members of the population, for example the perinatal and the immune compromised.

5.6. CONCLUSION

A higher risk of HUS cases per-year was estimated in raw than pasteurised PDBM. A higher risk of STEC infections was also observed in children below 5 years in comparison to the age group above 5 years. The model estimates show that the public health significance of HUS cases due to STEC contaminated PDBM depends on the current variability surrounding the risk profile of the milk and is explicitly influenced by consumer behaviour. Serving volumes, time taken to sell the milk at PD outlets and PDBM storage time at home had the greatest effect on the probability of HUS and the annual number of cases. A combination of PDBM handling practices (storage at 4°C throughout the whole chain, time taken to sell the milk (5 h) and time taken to consume the milk (half a day)) along the product value chain had more impact in reducing the risk of infection and probability of illness. Given that partitioning of milk also contributes to the risk of HUS, extreme caution needs to be taken during partitioning.

This study recommends strict enforcement of and adherence to SA Standard Code of Practice Food Hygiene Management (SABS 049), which regulates food hygiene in the dairy industry, especially for PDs. The current study also recommend the inclusion of, within the SABS 049, a specific guideline that regulates the production, processing and supply of PDBM. Furthermore, the training on dairy technology and safety for producers and suppliers of PDBM by the Department of Health, in collaboration with environmental health officers (in the different municipalities across SA) and non-governmental organisations, such as the Dairy Standard Agency needs to be strengthened to improve public health and safety. The raising of awareness on the health risks associated with the consumption of raw milk for, particularly, consumers of raw PDBM, also needs to be scaled-up for them to make informed decisions when buying milk. The awareness will indirectly encourage consumers to buy certified raw milk. Results from this study can be useful in formulating risk-

based mitigation strategies and policies. Additionally, the models developed in this study are an example of risk assessments for milk produced and marketed from similar scenarios.

CHAPTER SIX

GENERAL DISCUSSION

Food safety is a critical element towards the accomplishment of food and nutrition security and is essential in ensuring adequate safe food supply for consumers. Therefore, it is imperative to ensure that food is free from hazardous microbial, chemical and physical contaminants along the supply chain. Outbreaks of illnesses associated with the consumption of pathogen contaminated milk and its products are well documented and are still prominent to-date even in developed countries with adequate food safety and management systems in place (CDC, 2008; EFSA, 2015). However, food safety challenges faced, especially in developing countries, include lack of food safety knowledge, appropriate legislation and enforcement by respective governments (Uyttendaele et al., 2016). Milk and dairy products are income generating, raising billions of USD each year in revenue across the globe (Coetzee, 2014). The dairy industry today has established efficient production processes and wide product distribution networks, sometimes bringing with them unforeseen and adverse quality consequences to milk itself. The scale and complexities of today's dairy industry contribute to food safety challenges and increased likelihood and magnitude of foodborne illnesses (Ercsey-Ravasz et al., 2012). In this regard, there is a need to enforce strict and comprehensive quality regulations in the dairy industry, especially in developing countries, to protect public health (Yazdankhah et al., 2004). In actual fact, ensuring food safety in dairy is obligatory, not only to producers and retailers of milk, but it is also a responsibility of the consumer.

In SA, approximately 2% of the milk is produced, processed and sold directly to consumers by producers who are either unregistered or unauthorised and this milk is referred to as producer distributor bulk milk (PDBM). Studies on this milk have since found microbiological inadequacies in its quality (Jansen, 2003; Lues et al., 2003; O'Ferrall-Berndt, 2003; Cawe, 2006; Lues et al., 2010; Lues et al., 2012). In 2013, a survey on PDBM by a non-governmental organisation, the Dairy Standard Agency (DSA), in collaboration with environmental health officers (EHOs) (in the

different municipalities across SA) reported very high level counts of *E. coli*, more than stipulated in the Foodstuff, Cosmetics and Disinfectants Act (SA, 2001 Act (54), (1972)). In this context, a research project was funded by Milk South Africa (the dairy industry) to characterise the *E. coli* in PDBM and evaluate the risk associated with pathogenic *E. coli* if it is detected. Microbiological quality of PDBM samples from eight geographical regions in SA was determined. Enterobacteriaceae and other bacterial species in PDBM were characterised. *E. coli* was further characterised for the prevalence and distribution of virulence factors (*stx* 1, *stx* 2 and *hly*A), serotypes and antibiotic resistance patterns, which also included extended-spectrum β-lactamase (ESBL) producing capacity. Subsequently, the risk associated with the consumption of PDBM contaminated with the pathogenic *E. coli* was also assessed.

6.1. METHODOLOGICAL CONSIDERATIONS

Convenience sampling was used in this study to collect PDBM samples from different geographical regions across SA. Using this sampling method, PDBM samples were collected based on their availability in a particular region. A review by Keefe (1997) showed that studies on milk quality used the convenient sampling technique owing to the following advantages: cost effectiveness, simplicity of sampling and the ease of research and data collection can be facilitated in a short duration of time. The greatest weakness which was also a drawback in this study was the degree of generalisability and possible bias during sampling, which also invoked challenges during statistical analysis of results. Therefore, one of the intended objectives, the comparison of PDBM microbiological quality among the geographical regions could not be carried out due to huge differences in sample sizes across the regions. However, due to time and resource constraints, convenience sampling was the most appropriate in the current study. The more appropriate and widely applied approach when collecting milk samples for microbiological analysis in a survey of

this nature is the stratified random sampling technique. Notwithstanding being time consuming and tedious, this method ensures a high degree of representativeness in a population. Several studies have used stratified random sampling techniques in surveys of milk quality (Mühlherr et al., 2003; Chye et al., 2004; D'amico and Donnelly, 2010).

To evaluate the microbiological quality of PDBM, 3M Petrifilm plates were used for the enumeration of presumptive *E. coli* and other bacterial counts. 3M™ Petrifilm™ *E. coli*/coliform plates were used to detect and enumerate *E. coli* and coliforms. A pertifilm plate is a sample-ready-culture-medium system which contains Violet Red Bile (VRB) nutrients, a cold-water-soluble gelling agent, an indicator of glucuronidase activity (BCIG) and a tetrazolium indicator that facilitates colony enumeration in milk (Elumalai, 2016). Most *E. coli* (about 97%) produce beta-glucuronidase, which produces a blue precipitate associated with the colony. The 3M culture medium contains lactose which is fermented by *E. coli* (about 95%) and coliforms, thereby, producing a gas bubble which is trapped at the top of the film. *E. coli* and coliforms are identified as blue and red colonies, respectively, with gas bubbles.

Using this method, *E. coli* and coliforms were identified without subsequent confirmation steps, usually required with most traditional reference methods such as the membrane filtration or multiple fermentation tube methods based on most probable numbers (MPN) (Samarajeewa et al., 2010). By using the 3M plates, the amount of time consumed and possibility of contamination was reduced, which could be experienced when using traditional media preparation methods. Furthermore, less space was occupied by the plates and gave room for more samples to be analysed at a time.

The sensitivity of 3M *E. coli*/coliform plates is usually above 95% and the limit of detection for *E. coli* and coliforms is 1 cfu/ml (Beloti et al., 2003). 3M *E. coli*/coliform plates are validated for food and water samples (Priego et al., 2000). However, 3 to 5% of *E. coli* do not produce beta-glucuronidase or ferment lactose (Jefferson et al., 1986). Therefore, using 3M pertifilms, *E. coli* /coliform plate can be a setback as these strains, which do not produce beta-glucuronidase or ferment lactose, might not be recorded.

Potassium hydroxide (KOH) (3%) was used for Gram stain reactions (Romano et al., 2005). Conventional Gram staining is laborious and time consuming. This present study adopted the KOH method described by Halebian et al. (1981). KOH is a rapid, efficient and reliable method, especially for Gram-negative Enterobacteriaceae species (Powers, 1995). The principle behind the method is that Gram-negative bacterial cell wall lacks the peptidoglycan layer, which their counterparts Gram-positive, possess. Therefore, 3% KOH dissolve the cell wall of Gram-negative bacterial cells within seconds of application and not the Gram-positive cells. Dissolved cell wall releases the intercellular material and the liberated cellular DNA makes the mixture viscous or "stringy." The positive string test indicates a Gram negative organism without the time consuming part of staining and use of the microscope. Therefore, this method allowed us to reduce the amount of time for the Gram's reaction in this study.

Rapid species identification, strain diversity and source tracking of bacteria are required for mitigating food contamination. In this present study, MALDI-TOF/MS was applied in the characterisation of *E. coli* and other bacterial isolates to species level. Recent developments in mass spectroscopy have made it easy and possible to use whole-cell MALDI TOF/MS in the identification of microorganisms (Mazzeo et al., 2006; Pignone et al., 2006). It is an ideal method for measuring non-purified extracts and intact bacterial cells. MALDI TOF/MS relies on

proteomic profiling of ribosomal proteins, along with a few highly conserved proteins generated from direct ionisation of a colony of intact organisms or bacterial protein extract, and correlates this spectral signature to a database of spectra collected from reference strains (Maja et al., 2013). The MALDI-TOF/MS method is fast, accurate, less expensive than molecular and immunological-based detection methods and does not require trained laboratory personnel (Singhal et al., 2015). The method compares well with traditional PCR based methods, such as 16S and 18S in the identification of bacterial species (Maja et al., 2013). MALDI-TOF/MS has been applied successfully and accurately for clinical, food, water and environmental isolates (Singhal et al., 2015). However, disparities of the system on *S. aureus* have been noted; therefore the method need to be validated by use of traditional biochemical tests or PCR (Juiz et al., 2012). Other drawbacks of the method include: (i) reduced quality and reproducibility of bacterial MALDI-TOFMS fingerprints (ii) high initial capital and maintenance (Singhal et al., 2015).

In this study, *E. coli* isolates identified by the MALDI-TOF/MS were validated using PCR detection of the *uid*A gene. The *uid*A gene in *E. coli* encodes beta-glucuronidase (GUR), which is the first enzyme of the hexuronide-hexuronate pathway (Jefferson et al., 1986). The *E. coli* GUR-encoding *uid*A gene is conserved in the organisms and has been used as the target for *E. coli* detection (Bej et al., 1991). However, 1 to 3% of the *E. coli* lacks this gene (Jefferson et al., 1986). In view of this, to fully identify *E. coli*, a combination of biochemical, proteomic and molecular based methods were used in this study.

To evaluate the potential risk associated with consumption of PDBM, antibiotic resistance profiles of *E. coli* isolated from the milk were characterised. The motivation was that there is a growing concern of the transmission of antibiotic resistant bacteria through the food chain. The study evaluated resistance patterns of *E. coli* to common antimicrobials used (i) in human and animal

medicine, (ii) in prophylaxis, and (iii) as growth promoters. Furthermore, the ability of the isolates to produce ESBLs against cephalosporins and aztreonam was evaluated. The Kirby-Bauer disk diffusion method was used to elucidate resistance profiles of *E. coli* to common antimicrobials. Regulatory agencies and standards-writing organisations (the U.S. Food and Drug Administration (FDA) and the World Health Organisation (WHO)) published standardised reference procedures, based on the Kirby-Bauer method (Ericsson and Sherris, 1971). The Clinical and Laboratory Standards Institute (CLSI) adopted the Kirby-Bauer procedure as a consensus, which is periodically updated as further emerging resistance scenarios are experienced (CLSI, 2011). The aforementioned organisations recommend Mueller Hinton Agar for disk diffusion testing of non-fastidious organisms such as Enterobacteriaceae. The disk diffusion antibiogram provides qualitative results by categorising bacteria as susceptible, intermediate or resistant. However, when quantitative information is required, the dilution method is appropriate for the determination of minimum inhibitory concentration (MIC) values (Balouiri et al., 2016).

Screening for ESBLs in *E. coli* was carried out using the modified double disk diffusion method on Mueller Hinton Agar. The method has been applied successfully on *E. coli* isolates from different environments, which include food (Pitout et al., 2003; Njage and Buys, 2015). ESBLs are enzymes that mediate resistance to extended-spectrum (third generation) cephalosporins (e.g., ceftazidime, cefotaxime, and ceftriaxone) and monobactams (e.g., aztreonam). Different methods have been applied for screening ESBLs in *E. coli*, although the most appropriate way is to combine both phenotypic and genotypic characterisation. In this study, only phenotypic characterisation for ESBLs in *E. coli* was applied. Determinants of ESBLs in *E. coli* are plasmid mediated and might not be expressed on different growth media, which subsequently give rise to false negatives (Overdevest et al., 2011).

A method by Coimbra et al. (2000), which uses restriction length polymorphism (RFLP), was adopted, modified and used for serotyping the *E. coli* isolates. Genotypic data (as articulated by Coimbra et al. (2000) and phenotypic data (serum agglutination) were integrated. The method demonstrated cost effectiveness and was less time intensive and improved the possibility of detecting the often minimal amount of variation between strains. Conventional serotyping, using serum agglutination is very expensive and need referral laboratories, which are very few around the world (Coimbra et al., 2000). Other phenotypic methods, including bacteriophage typing, have been used for many years, although these methods are generally time consuming and not always accurate (Fratamico et al., 2016). Molecular based techniques have been developed, which target the O and H gene clusters (Fratamico et al., 2016). The genes that encode for O-antigens are located on a chromosome in a cluster designated as the O-antigen gene cluster (O-AGC). These are flanked by two conserved sequences called *JUMP*start, a 39 bp-element at the 5′ end (Wang and Reeves, 1998), which is downstream of *gal*F (UTP-glucose-1-phosphate uridylyltransferase) and *gnd* (6-phosphogluconate dehydrogenase) at the 3′ end. Analysis of the O-AGCs of all *E. coli* O-groups showed that the sizes of the O-AGCs and their gene content vary considerably, which results in the variability of O-antigens (Coimbra et al., 2000). Fratamico et al. (2016) reviewed molecular based methods such as multiplex PCR, high throughput real-time PCR and whole genome sequencing and SNP analysis used in serotyping *E. coli*. Despite being efficient, these methods proved to be laborious and expensive.

In this study, the O-AGC (*rfb*) was amplified prior to restriction digestion using restriction enzyme *Mbo*II, as described by Coimbra et al. (2000). As a result of the plasticity of the O-antigen genes in *E. coli* and more so, the diversity of the genetic pool in different geographical regions, the O-AGC band size and the resulting bands from restriction digestion, did not match what was

described by Coimbra et al. (2000). Therefore, the method was modified at this level. This method was used based on the fact that clustering RFLP patterns (generated from conserved regions in bacteria) groups similar serotypes into unique clusters. However, other methods for *E. coli* clustering such as GTG_5 and pulsed-field gel electrophoresis (PFGE) are also possible options. The serotyping method that was used in the current study is fast, cost effective (since the cost and time of serum agglutination are reduced) and reproducible. Nonetheless, it has some setbacks when serum agglutination cannot deduce the serotype due to autoagglutination. Therefore, in such cases, earlier articulated molecular based methods are ideal.

Phenotypic and molecular based methods were used to detect STEC in this study. However, one very important limitation was in the method (3M pertifilm plates), which was used to isolate the *E. coli*. STEC cells are known to enter a dormancy state in food, which includes milk, where they remain viable but non-culturable (Dinu and Bach, 2011). Therefore, this greatly underestimated the quantities of STEC in milk, although viability and pathogenicity can be retained. The most sensitive method for STEC isolation, detection and quantification in milk is the immunomagnetic separation, following selective enrichment, and subsequent spread-plating of the concentrated target cells onto STEC chromagar (Boer and Heuvelink, 2000). Thereafter, molecular screening of the *stx* genes in the phenotypic positive isolates can follow, for validation (Trevisani et al., 2013).

STEC isolates, in this study, were first screened on three different selective media to increase chances of detection and isolation. *Stx* virulence factors were identified, first, by screening using a multiplex real-time PCR kit from Bio-Rad. This method was used successfully by Aijuka et al., (2015). However, this method does not determine the type of *stx* gene present in the *E. coli*. Therefore, a multiplex PCR method was then applied, published by Cebula et al. (1995), to detect

the type and prevalence of *stx* genes present in the *E. coli* serotypes. Several studies on *E. coli* from different sources have characterised *stx* genes successfully using the method explained by Cebula et al. (1995). Although the detection of an encoding gene does not translate to the expression or production of the toxin, both molecular and phenotypic techniques are still widely used to give an indication of the potential hazard in food.

Presence of haemolysis in *E. coli* was detected using both genotypic and molecular characterisation. Alpha-haemolysis in *E. coli* isolates was observed. These isolates were positive for haemolytic expression on blood agar, however, only *hly*A gene was then screened. It is important to fully understand the haemolytic characteristic of *E. coli* by genotypic detection of all the haemolytic genes, *she*A, *ehx*A and *e-hly*A.

To determine the relationship of *E. coli* isolates from different geographical regions, molecular (GTG_5), phenotypic (antibiograms profiles) and proteomics (MALDI-TOF/MS) based finger prints were used. ClinProtTools software was used to cluster peptide mass fingerprints (PMF) generated by the MALDI-TOF/MS for each *E. coli* isolate. Studies have successfully applied this software to discriminate *E. coli* isolates from different environments, as well as in bacterial source tracking (Maja et al., 2013; Singhal et al., 2015). GTG_5 fingerprinting is easy to perform, based on primers complementary to certain repetitive sequences dispersed in bacterial genomes (Versalovic et al., 1994). Clusters of *E. coli* were generated from antibiogram profiles using XLSTAT. Antibiogram patterns have been used to discriminate *E. coli* and also in tracking its source in the environment (Nontongana et al., 2014). Both molecular and proteomics based clustering of *E. coli* isolates gave similar trends of clustering (Ntuli et al., 2016; 2017). Other techniques can be applied to distinguish *E. coli* and these include 16S sequencing, randomly amplified polymorphic DNA (RAPD) and the multilocus sequencing technique that focuses on sequencing housekeeping genes,

although they are time consuming and expensive. The methods used in the study for discriminating *E. coli* are reproducible, reliable, cost effective and less time consuming.

A modular process risk assessment (MPRA) method was used (Nauta, 2002) to estimate the burden of illnesses associated with the consumption of STEC contaminated PDBM. The method was adopted by international organisations, such as CAC, FAO, EFSA and CDC and several researchers have used MPRA to estimate the risk of STEC in milk (Grace et al., 2008; Giacometti et al., 2012a; Giacometti et al., 2016). However, there was a lot of variability and uncertainty in data inputs that was used in the developed model in this study. Therefore, to gain an insight on the actual risk of illnesses caused by STEC in PDBM, more data is needed to reduce the degree of uncertainty.

6.2. CHARACTERISATION OF *E. COLI* AND OTHER ENTEROBACTERIACEAE SPECIES

The microbiological quality of PDBM collected from outlets revealed the impacts of milk production, storage, processing, handling and distribution by PDs. Results from the study suggest unsanitary conditions/practices during production, processing, distribution or storage of PDBM and this also affects its shelf-life. The quality of PDBM in SA has always been highlighted as a public health concern, as shown by various studies (Jansen, 2003; Lues et al., 2003; O'Ferrall-Berndt, 2003; Cawe, 2006; Agenbag, 2008; Lues et al., 2010; Caine et al., 2014; Msolo, 2016). As reported by Agenbag (2008), PDs focus more on volumes of milk produced and very little or no attention is given on its quality. Furthermore, there are no penalties for poor quality milk under this supply chain. Many PDs do not have sufficient technical and scientific knowledge, in both dairy science and dairy microbiology, for large scale collection and distribution of saleable milk

(O'Ferrall-Berndt, 2003). They often produce and distribute milk without any knowledge of milk hygiene or dairy technology and the associated health risks of milk contaminated by pathogens. This is evident from the current study where the average storage conditions and time taken to sell the milk at PD outlets were recorded. Failure to maintain a cold chain and taking more time to sell the milk as noted in Chapter 4 has been implicated in the proliferation of microbial growth, subsequently giving rise to a high prevalence of *E. coli*, coliforms and other microorganisms in the milk (Latorre et al., 2011).

The rate at which milk quality declines depends on extrinsic and intrinsic factors at farm level (EFSA, 2015), as well as the temperature and amount of time that milk is stored before selling to the consumer or treatment at a factory. Factors giving rise to extrinsic contamination include: the poor hygiene of the milker, unclean animals and equipment, poor feeding and housing strategies and contaminated rinsing water for milking machine and equipment (Reinemann et al., 2003; Torkar and Teger, 2008; Ntuli et al., 2016). Contamination, which can arise from systematic infections, as well as mastitis, is referred to as intrinsic contamination.

This research study recorded the presence of alkaline phosphatase in 21% (n=104) of pasteurised PDBM samples (Ntuli et al., 2016). The research also noted that 36.6, 92.1 and 58.0% of pasteurised PDBM samples from all the eight provinces were above the minimum standards prescribed in the Foodstuffs, Cosmetics and Disinfectants Act (Act 54 of 1972), with respect to *E. coli*, coliforms and total plate count, respectively. In this view, significant percentages of pasteurised milk enter the market without being able to pass the alkaline phosphatase test. Pasteurised PDBM can be contaminated after processing and this is undoubtedly as a result of poor post processing milk handling practices. Bacteria recontamination of pasteurised milk originate primarily from water aerosols and air in the filling equipment or improperly cleaned processing

and handling equipment (Eneroth et al., 2000; Ledenbach and Marshall, 2010). Recently, certain species of bacteria (vegetative form) have been found to harbour heat resistant genetic determinants and this can suggest the cause of contamination in pasteurised PDBM (Mercer et al., 2015). Contamination of pasteurised milk by raw milk is also a source of bacterial recontamination in processed milk.

Findings from this study revealed a diversity of Enterobacteriaceae and other bacterial species from both raw and pasteurised PDBM. The most prevalent Enterobacteriaceae species, in decreasing order, were *E. coli, R. ornithinolytica, K. oxytoca, E. cloacae, E. asburiae, S. liquefaciens* and *H. alvei.* Bacteria, such as *Pseudomonas* spp., which are implicated in dairy spoilage, were frequently isolated in PDBM from all the provinces (Table 3.1). Bacteria in bulk tank milk have been observed to consist of a variety of bacterial species, which is indicative of their origin or source. The current study could not establish the possible source and pathway of bacteria species in pasteurised PDBM. Many of the species isolated in pasteurised PDBM were waterborne (which belonged to the following genera; *Acinetobacter, Aeromonas, Buttiauxella, Citrobacter, Pseudomonas, Raoultella* and *Serratia*), suggesting a potential cross-contamination from processing environments, arising from water aerosols and improperly cleaned processing and handling equipment (Eneroth et al., 2000). Appropriate handling of pasteurised milk certainly reduces milk and dairy spoilage, ultimately extending milk shelf-life. Furthermore, this reduces the risk to the consumer that can be posed by pathogenic microorganisms in milk. Bacterial species (i.e. *Y. enterocolitica, E. cloacae* and *E. asburiae*), which have been implicated in milkborne disease outbreaks and are considered health hazards (Boerlin et al., 2001; Oliver et al., 2009) were isolated in the current study, suggesting that PDBM can be a potential public health risk to consumers.

E. coli was detected in 36% of PDBM samples and in six out of eight provinces in SA. The presence of *E. coli* in raw milk can be via intramammary secretion or via faecal contamination of the udder or milking equipment. Considering milk and dairy processing, *E. coli* is a faecal indicator organism, whose presence in milk suggests that other organisms of faecal origin, including pathogens such as *Salmonella* spp., *Y. enterocolitica*, *L. monocytogenes* and *Campylobacter* spp., may also be present.

PDBM samples with antimicrobial residues were detected in this study. The risk of exposure to inhibitory substances such as antimicrobials in milk had been reported earlier (Kurwijila et al., 2006). Although some scholars have conducted studies, which suggest a link between the emergence of resistance in bacteria and the use of antimicrobials as growth promoters in animal feeds or for treatment, a consensus is still lacking. Safety concerns have been raised in cases where drug resistant bacteria are isolated from food because of the potential transfer of resistant foodborne pathogens to humans through the food chain (Moyane et al., 2013).

More than 75% (n=121) of the isolated *E. coli* was resistant to the common antibiotics used in agriculture (oxytetracycline, cephalothin, nitrofurantoin, amoxicillin, ampicillin, neomycin, amikacin, gentamicin, nalidixic acid, chloramphenicol and norfloxacin). Most of these antibiotics are used in SA for treatment in the medical and agricultural sectors, as prophylaxis or feed additives to promote growth in animals (Moyane et al., 2013). Further characterisation of the *E. coli* revealed that a significant number (20.6%) had extended-spectrum β-lactamase (ESBL) producing capacity to cerphalosporins and aztreonam. ESBL producing Enterobacteriaceae have been identified in food animals and very few in products such as milk, unless the isolates came from milk obtained from mastitic cows (Geser et al., 2012). Some of the *E. coli* isolated (60%) confirmed multidrug resistance (MDR). In this study, similar antibiotic resistant patterns were

observed in *E. coli* isolates from all the provinces in SA, suggesting exposure to similar antibiotic use despite the geographical differences. This was also evident from cluster analysis using antibiotic profiles of the *E. coli* isolates (Figure 4.5).

The study also detected *E. coli* with virulence factors (*stx* and *hyl*A), which have been associated with strains belonging to the enterohemorrhagic *E. coli* (EHEC) serotype. The presence of virulence factors linked to EHEC in PDBM suggests the ability to cause shigatoxin-related human infection and disease. EHEC, particularly STEC O157 serotype, have been implicated in many foodborne disease outbreaks across the globe, although STEC non-O157 serotypes are also of important public health significance. Recent outbreaks caused by STEC serotypes in milk are well reported and documented (CDC, 2005; Oliver et al., 2009; EFSA, 2015). STEC with the ability to cause haemolysis, MDR capacity and capable of producing ESBLs were isolated from PDBM (Ntuli et al., 2016; 2017). A correlation between virulence factors and antibiotic resistant genes in *E. coli* has been reported. The dissemination of resistance is associated with genetic mobile elements, such pathogenicity islands that may also carry virulence determinants (da Silva and Mendonça, 2012). Apart from the virulence traits and antibiotic resistant genes being on the same genetic island as plasmids, *E. coli* can also acquire virulence factor-encoding prophase (on chromosomal DNA) or plasmid bearing antibiotic resistance factors (Rasko et al., 2011). This is a cause of concern as most infections from such pathogenic *E. coli* become difficult to treat, subsequently increasing the burden of illness. PDBM, in this study, may serve as a reservoir of antibiotic-resistant pathogens and can pose a food safety risk to the public as new antibiotic resistant pathogenic Enterobacteriaceae strains can emerge through gene transfer.

Previous studies have focused on the eminent STEC O157 in milk, but in this research study, the emphasis was on both O157 and non O157 STEC seropathotypes in PDBM. The European Food

Safety Authority (EFSA) and the U.S. Department of Agriculture (USDA) have issued recommendations for laboratory testing for these pathogens (Eblen, 2007; EFSA, 2007). Fifteen different *E. coli* O-serogroups were detected (O2, O4, O9, O20, O43, O64, O68, O83, O85, O109, O112, O119, O147, O155 and O157) from PDBM (Ntuli et al., 2017). The dominant serotype was O157. STEC non-O157 serogroups reported in the current study were different in comparison to the commonly isolated STEC non-O157 (O111, O26, O103, and O145) serogroups from foods such as milk in continental Europe, USA, Japan and Canada (Eklund et al., 2001). However, similar serotypes found in the present study have previously been isolated from cattle and humans; of which 64.0% of the serotypes were commonly isolated from humans, suggesting cross contamination during the milk value chain (Constantiniu, 2002).

A higher proportion of *stx* 2 genes than *stx* 1 among the STEC serotypes were reported in this present study (Ntuli et al., 2017). *Stx* 2 gene is reported to produce more virulent shigatoxin cytotoxin in humans compared to *stx* 1 (Karch et al., 2005). Therefore, the estimated burden of illness reported in the current study can be fatal as a result of this. Despite *E. coli* strains belonging to other serogroups (diarrheagenic *E. coli*) of public health concern having been detected in the bulk milk in SA, only EHEC seropathotypes were investigated in this study (Caine et al., 2014). Cluster analysis revealed that, although phenotypically similar, the *E. coli* seropathotypes from the different geographical regions are genetically diverse. These variations among *E. coli* pathotypes are important for epidemiological and clinical purposes (Khan et al., 2002).

6.3. QUANTITATIVE MICROBIAL RISK ASSESSMENT FOR STEC IN PDBM

This study also estimated the nationwide PDBM scenario of HUS cases that may be linked to the consumption of STEC contaminated PDBM. Prevalence of STEC in PDBM was found to be very

low. Nevertheless, when taking careful consideration, the low infection dose of this pathogen (about 100 to 200 or even less than 10 cells in susceptible individuals) is still a major public health-risk (Nguyen and Sperandio, 2012; Dean et al., 2013). A higher risk of HUS cases per year was recorded in raw than pasteurised PDBM and also in age groups below 5 years.

Pasteurisation is one of the most approved methods by international laws to control and reduce pathogenic infections from milk. This study could not establish the possible source and pathway of STEC in pasteurised PDBM, although this was explained as either inadequate pasteurisation process or contamination/cross-contamination of a batch of PDBM after a successful pasteurisation (Ntuli et al., 2016). The risk is dependent on the variability surrounding the risk profile of the milk and is explicitly influenced by consumer behaviour. The risk associated with *E. coli* seropathotypes can be complicated given that the isolates also possessed MDR and ESBL producing capacity. Poor PDBM quality as found in this study, attributed to poor handling of PDBM along the value chain, is suggested to pose the risk associated with STEC in PDBM. The presumptive route by which STEC enters milk is via faecal contamination and intramammary secretions (Farrokh et al., 2013). *E. coli* can grow under improper storage conditions. In view of this, reducing HUS cases starts at the farm where good agricultural practices need to be implemented.

PDBM in SA support both rural and peri-urban communities in different geographical regions. It remains as a source of income and social welfare for PDs, despite their decreasing numbers. However, the findings presented in this study raise a concern from a public health stand-point due to public health risk posed, especially to consumers of PDBM who are poor and vulnerable.

6.4. POTENTIAL FOR FUTURE STUDY

The study characterised STEC serogroups, which were found in PDBM form different provinces in SA and also estimated the resultant burden of illnesses. Therefore, it is necessary to characterise other diarrheagenic *E. coli* serotypes in PDBM so as to fully understand the risk posed by these pathogens in the milk. The study did not detect *eae* gene in STEC, which also included O157. It is important to investigate other factors mediating virulence in *E. coli* in order to elucidate its pathogenicity. Antibiotic profiles of *E. coli* were detected using phenotypic based methods. In view of this, a future research can be designed towards elucidating and establishing the presence and distribution of resistant genetic marker(s) in the phenotypically resistant *E. coli* isolates using molecular based techniques. Furthermore, characterisation of plasmids in *E. coli* responsible for ESBL producing capacity is also necessary. As a result of concerns raised about the potential transfer of antibiotic resistant gene to humans through the food chain, studying the acquisition of antibiotic resistant gene in food environment, such as milk can be designed in future to fully understand the risk posed to consumers. New molecular based methods (such as new generation sequencing) and traditional phenotypic typing of *E. coli* can be employed to determine its exact source and origin in the PDBM supply chain.

The study also revealed that PDBM was contaminated with other bacterial species. Milkborne diseases are not caused by only pathogenic *E. coli* in food across the globe. Therefore, it is imperative to investigate the main microbial hazards recognised as milkborne pathogens (*Salmonella* spp., *L. monocytogenes, S. aureus, Campylobacter* spp.) in PDBM and also to conduct risk assessments for each of them. Hence, more data is needed on the actual scenario of PDBM production, processing, storage, distribution, handling and consumption patterns at the consumer

level in order to conduct a comprehensive risk assessment associated with pathogen contaminated PDBM in SA.

CHAPTER SEVEN

CONCLUSIONS AND RECOMMENDATIONS

This study reports poor microbiological quality of PDBM in SA, which is characterised by the occurrence of high microbial counts, with subsequent reduction in the shelf life of the milk. The milk was found to be a reservoir of pathogenic bacteria that potentially pose public health risks to consumers. A wide diversity of *E. coli* (seropathotypes with different virulence factors) and Enterobacteriaceae species belonging mostly to spoilage microbiota were detected in PDBM. Both raw and pasteurised retail PDBM was found to harbour multidrug resistant pathogenic *E. coli*, which had the capacity to produce ESBLs that may hydrolise cephalosporins. This is a cause of concern as the strains are a potential source of antimicrobial resistant foodborne pathogens in humans through the food chain. Detection of *E. coli* serotypes, in PDBM, carrying virulence genes that are known to cause human diseases and are also resistant to antibiotics can present a significant public health risk, especially to the vulnerable members of the community. This study highlights a potential risk posed to human health by consuming PDBM contaminated by STEC.

The risk assessment revealed that a higher risk of HUS cases per-year was estimated in raw than pasteurised PDBM. A higher risk of STEC infections in the age group below 5 years was also observed. The model estimates show that the current variability surrounding the risk profile of the PDBM, which is influenced by consumer behaviour, influences the public health significance of HUS cases due to STEC contaminated milk. Good handling practices of PDBM along the product value chain had a positive impact in reducing the risk of infection and probability of illnesses.

This study recommends strict enforcement of and adherence to SA Standard Code of Practice Food Hygiene Management (SABS 049), which regulates food hygiene in the dairy industry, especially for PDs. The current study recommends the inclusion of, within the SABS 049, a specific guideline that regulates the production, processing and supply of PDBM. Furthermore, the training on dairy technology and safety for producers and suppliers of PDBM by the Department of Health, in

collaboration with environmental health officers (in the different municipalities across SA) and non-governmental organisations, such as the Dairy Standard Agency, needs to be strengthened to improve public health and safety. The raising of awareness on the health risks associated with the consumption of raw milk for, particularly, consumers of raw PDBM, also needs to be scaled-up for them to make informed decisions when buying milk. The awareness will indirectly encourage consumers to buy certified raw milk. Figure 7.1 presents a collaborative effort on how academia, industry, non-governmental organisations and the government, can improve public health and safety associated with STEC in PDBM.

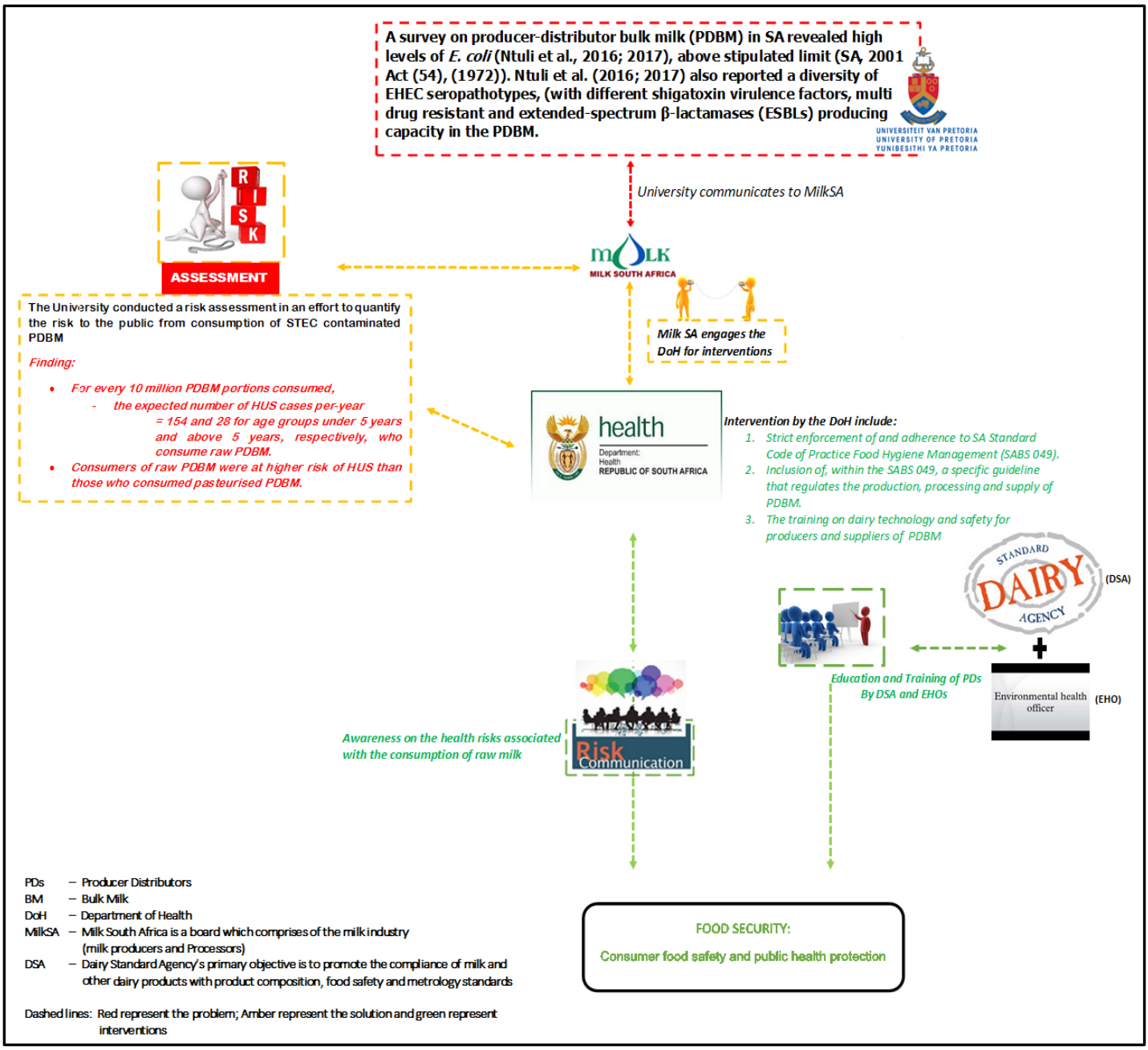

Figure 7.1: A collaborative effort of academia, industry, non-governmental organisation and the government to improve public health and safety associated with Shigatoxin producing *E. coli* in producer – distributor bulk milk.

CHAPTER EIGHT

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CHAPTER NINE

PUBLICATIONS

9.1. REFEREED JOURNAL ARTICLES

Ntuli, V., Njage, P.M.K & Buys, E.M. 2016. Characterisation of *Escherichia coli* and other Enterobacteriaceae in producer-distributor bulk milk. *Journal of Dairy Science*, 99**,** 9534-9549.

Ntuli, V., Njage, P. M. K & Buys, E. M. 2017. Extended-spectrum β-lactamase, shigatoxin and haemolysis capacity of O157 and non-O157 *E. coli* serotypes from producer-distributor bulk milk. *International Dairy Journal,* 66**,** 126-134.

9.2. PRESENTATIONS

9.2.1. Oral

Ntuli Victor and Elna Buys (2017). 'Quantitative risk assessment for shigatoxin producing *E. coli* in bulk milk sold directly from producer to consumer'. 50th Dairry AGM/Symposium, 'Dare to Dairy'. Organised by SASDT, (8-11 May 2017) KIEVITS KROON, Pretoria, South Africa

Ntuli Victor, Patrick Njage and Elna Buys, (2015). 'Antibiotic resistance patterns and virulence factors of O157 and non-O157 *E. coli* serotypes in milk'. 21th SAAFoST Biennial Congress & Exhibition. Southern Sun Elangeni & Maharani Beachfront Hotels in Durban, KZN, South Africa. 6-9 & 10 September 2015.

Ntuli Victor, Patrick Njage and Elna Buys, (2015). 'ANTIBIOTIC RESISTANCE PATTERNS OF IDENTIFIED *E. COLI* SEROTYPES IN BULK MILK'. 48th Dairy AGM/Symposium, 'Innovationand Cost Optimisation'. Organised by SASDT, (14-16 April 2015) Cape St Francis Resort, Cape St Francis, Eastern Cape, South Africa

Ntuli Victor, Patric Njage and Elna Buys, (2014). 'Prevalence and characterisation of Enterobacteriaceae in milk'. 47th Dairy AGM/Symposium, 'INTEGRATED SUPPLY CHAIN

MANAGEMENT'. Organised by SASDT, (22-26 April 2014) Lagoon Beach Hotel Cape Town, South Africa

9.2.2. Posters

Ntuli Victor and Elna Buys, (2016). 'Potential public health risk associated with multidrug resistant shigatoxin producing *E. coli* (STEC) O157 and non-O157 from producer-distributor bulk milk'. 18th World Congress of Food Science and Technology. International Union of Food Science and Technology (IUFoST) (21-25 July 2016), Dublin, Ireland.

Ntuli Victor, Patrick Njage and Elna Buys, (2016). 'Quantitative risk assessment for shigatoxin producing *E. coli* (STEC) in raw and pasteurised bulk milk sold directly from producer to consumer in the informal market in South Africa'. 25th International ICFMH conference, FOOD MICRO (19-22 July 2016), Dublin, Ireland.

Ntuli Victor, Patrick Njage and Elna Buys, (2015). 'Characterisation of STEC in bulk milk'. International Dairy Federation (IDF) World Dairy Summit, Vilnius, Lithuania, 20 – 24 September, 2015.

Ntuli Victor, Patric Njage and Elna Buys, 'Characterisation of STEC in milk'. International Association for Food Protection (IAFP) 2015. (25 – 28 July 2015). Oregon Convention Center in Portland, Oregon, USA.

Ntuli Victor, Patric Njage and Elna Buys, (2015). 'Antibiotic resistance profiles of identified *E. coli* serotypes in milk', The national University of Lesotho's 1st Annual Science & Technology Innovation Summit/Exhibition, Maseru, January, 2015.

Ntuli Victor, Patric Njage and Elna Buys, (2014). 'Characterisation and source tracking of *E. coli* in milk'. 24th International ICFMH conference, FOOD MICRO (1-4 September 2014), Nantes, France.

Ntuli Victor, Patrick Njage and Elna Buys, (2013). 'Prevalence and characterisation of Enterobacteriaceae in Bulk Milk'. 20th SAAFoST Biennial Congress & Exhibition and ICMSF Workshop CSIR, International Convention Centre, Pretoria, South Africa. 7-9 & 10 October 2013.