DETERMINATION OF THE MICROBIOLOGICAL SAFETY OF SELECTED FRESH PRODUCE OF INFORMAL RETAILERS POINT-OF-SALE

by

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

The global consumption of fresh produce has increased as consumers have become more health conscious. With the rise of fresh produce consumption, fresh produce related foodborne outbreaks also increased globally. Recent outbreaks have included the *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella* infections caused by contaminated fresh produce in 2018, 2016 and 2015, respectively. To minimise the risk for foodborne outbreaks in fresh produce it is important to know the current microbiological safety status of fresh produce in South Africa. Limited information is available about the microbiological safety of fresh produce sold at informal markets. Fresh produce is often consumed raw and therefore the microbiological risk is higher. A group of environmental bacteria, the Extended Spectrum β-Lactamases (ESBL) producing *Enterobacteriaceae*, are also of concern because of their ability to counteract the effect of antibiotics and spread to the environment and fresh produce.

The aim of this study was to determine the microbiological safety of fresh produce sold at the informal market in the Cape Town Metropolitan area, South Africa, by enumerating hygiene indicator systems such as coliforms, *E. coli* and *Enterobacteriaceae*. Indicator systems, however, do not give an indication of the presence of specific pathogens. The presence of produce-related pathogens such as *Salmonella*, *Listeria monocytogenes* and Shiga Toxin-producing *E. coli* (STEC) were also investigated. Also included in this study was the detection of Extended Spectrum β-Lactamase (ESBL) producing bacteria and their antibiotic resistance profiles.

Five informal vendors were selected to represent the informal market in the Cape Town metropolitan area. Each site was visited three times and at each site, two different products were selected for sampling (five replicates of each product). The fresh produce tested in this study included lettuce, cabbage, spinach, tomatoes, green beans and green peppers.

The general hygiene counts for all sites were well over the advised coliform limits according to the Department of Health. No *Salmonella* or *Listeria monocytogenes* was detected in any of the fresh produce. The presence of *E. coli* occurred in sporadic cases indicating evidence of poor handling practices at the informal vendors. The prevalence of ESBL producing *Enterobacteriaceae* was relatively low with 4% of the fresh produce sampled that tested positive for ESBL producing *Enterobacteriaceae*. Multiplex polymerase chain reaction (PCR) was used to confirm the presence of the most prevalent ESBL genes in an isolate namely bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{SHV}. Out of the seven phenotypically confirmed ESBL producing *Enterobacteriaceae,* five isolates were confirmed as containing at least one of the ESBL genes of interest. All ESBL producing *Enterobacteriaceae* were multidrug resistant as well, being resistant to at least Ampicillin, Cloxacillin and/or Cefoxitin, Tetracycline, Ciprofloxacin and Trimethoprim-sulfamethoxazole. Taking all the evidence into consideration, it is clear that post-harvest handling of fresh produce can be improved. In this study, the presence of ESBL producing *Enterobacteriaceae* on fresh produce has been confirmed in

samples sold at informal markets in the Cape Town metropolitan area. It is therefore recommended that the prevalence of these organisms is further monitored in the future.

UITTREKSEL

Die wêreldwye verbruik van vars produkte het toegeneem namate verbruikers meer gesondheidsbewus geword het. Met die toename in varsprodukte inname het varsprodukte verwante voedsel-uitbrake ook wêreldwyd toegeneem. Onlangse uitbrake sluit in die *E. coli* O157:H7, *Listeria monocytogenes* en *Salmonella* besmette produkte wat onderskeidelik in 2018, 2016 en 2015 plaas gevind het. Om die risiko van uitbrake in vars produkte te verminder, is dit belangrik om die huidige mikrobiologiese veiligheidstatus van vars produkte in Suid-Afrika te monitor. Beperkte inligting is beskikbaar oor die mikrobiologiese veiligheid van vars produkte wat verkoop word in informele markte in Suid Afrika. Varsprodukte word dikwels rou geëet en daarom is die mikrobiologiese risiko hoër. 'n Groep omgewingsbakterieë, Extended Spectrum β-Lactamase (ESBL) produserende *Enterobacteriaceae*, is ‘n kommer weens hul vermoë om die effek van antibiotika teen te werk wanneer ‘n individu geinfekteer word as gevolg van die verspreiding na die omgewing asook vars produkte.

Die doel van hierdie studie was om die mikrobiologiese veiligheid van vars produkte wat in informele markte in die Kaapstadse metropolitaanse gebied, Suid-Afrika, verkoop word, te bepaal. Dit is gedoen deur higiëne-aanwysersisteme soos coliforme, *E. coli* en *Enterobacteriaceae* te bepaal. Higiëne-aanwysersisteme gee egter nie aanduiding van die teenwoordigheid van spesifieke patogene nie. Die teenwoordigheid van patogene, algemeen teenwoordig in varsprodukte, naamlik *Salmonella*, *Listeria monocytogenes* en Shiga Toxin-produserende *E. coli* (STEC) is ook bepaal. Ook ingesluit in hierdie studie was die deteksie van ESBL produserende bakterieë en hul antibiotiese weerstandsprofiele.

Vyf informele verkopers is gekies om die informele mark in die Kaapstadse metropolitaanse gebied te verteenwoordig. Elke mark is drie keer besoek. By elke mark is twee verskillende produkte gekies waar vyf replikate van elke produk geneem is. Die vars produkte wat in hierdie studie getoets is, sluit in blaarslaai, kool, spinasie, tamaties, groenbone en groen soetrissies.

Die algemene higiëne tellings vir meeste produkte by al die markte was oor die geadviseerde coliforme limiete volgens die Departement van Gesondheid. Geen *Salmonella* of *Listeria monocytogenes* is opgespoor in enige van die vars produkte nie. Die teenwoordigheid van *E. coli* het voorgekom in sporadiese gevalle, wat bewys lewer van swak hanteringspraktyke by die informele verkopers. Die voorkoms van ESBL produserende *Enterobacteriaceae*, was relatief laag met 4% van die varsprodukte wat positief getoets het vir ESBL produserende *Enterobacteriaceae*. Polimerase Kettingreaksie (PKR) is gebruik om die teenwoordigheid van die mees algemene ESBL gene in 'n isolaat te bevestig naamlik bla_{TEM}, bla_{CTX-M} en bla_{SHV}. Van die sewe fenotipes bevestigde ESBL produserende *Enterobacteriaceae*, is vyf isolate bevestig wat ten minste een van die ESBL-gene bevat het. Alle ESBL produserende *Enterobacteriaceae* was ook weerstandig teen drie of meer antibiotikas insluitend Ampicillin, Cloxacillin en/ of Cefoxitin, Tetracycline, Ciprofloxacin en Trimethoprim-sulfamethoxazole. Gedurende die studie, is dit duidelik dat na-oes hantering van vars

produkte verbeter kan word. In hierdie studie is die teenwoordigheid van ESBL produserende *Enterobacteriaceae* op vars produkte wat verkoop word by informele markte in die Kaapstadse metropolitaansegebied, Suid Afrika bevestig. Daar word dus aanbeveel dat die teenwoordigheid van hierdie organismes in die toekoms verder gemonitor moet word.

This thesis is dedicated to

יהוה, my God,

who rule over me, for guidance, grace

and perseverance in every moment,

which without, nothing is possible.

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This thesis is presented in the format prescribed by the Department of Food Science at Stellenbosch University. The structure is in the form of one or more research chapters (papers prepared for publication) and is prefaced by an introduction chapter with the study objectives, followed by a literature review chapter and culminating with a chapter for elaborating a general discussion and conclusion. Language, style and referencing format used are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

LIST OF ABBREVIATIONS

AFNOR	Association Française de Normalisation
AST	Antibiotic Susceptibility Testing
ATCC	American Type Culture Collection
BPW	Buffered Peptone Water
CDC	Centre of Disease Control
CFU	Colony forming units
CLSI	Clinical & Laboratory Standards Institute
CTSS	Cape Town Scientific Service
DAEC	Diffusely Adherent *E. coli*
DAFF	Department of Agriculture, Forestry and Fisheries
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DoH	Department of Health
dsDNA	Double-stranded Deoxyribonucleic acid
EAggEC	Enteroaggregative *E. coli*
EC-broth	*E. coli* broth
EE-broth	*Enterobacteriaceae* Enrichment broth
EFSA	European Food Safety Authority
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
EPS	Extracellular polymeric substances
ESBL	Extended Spectrum Beta-Lactamase
ETEC	Enterotoxigenic *E. coli*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agricultural Organisation
FDA-BAM	Food and Drug Administration Bacteriological Analytical Manual
GAP	Good Agricultural Practices
HIV	Human Immunodeficiency Virus
HUS	Haemolytic-uremic Syndrome
ICU	Intensive Care Unit
ISO	International Organization for Standardization
L-EMB	Levine Eosin-Methylene Blue Agar
MALDI-ToF	Matrix-assisted Laser Desorption/Ionization Time-of-Flight

MDR	Multidrug Resistant
MPN	Most Probable Number
mRNA	Messenger Ribonucleic acid
NA	Nutrient Agar
NF ISO	“AFNOR validation mark”
OD	Optical Density
PABA	Para-aminobenzoic acid
PBPs	Penicillin-binding Proteins
PCR	Polymerase Chain Reaction
R	Resistant
RNA	Ribonucleic acid
RTE	Ready-to-eat
RV-broth	Rappaport Vassiliadis broth
S	Susceptible
SA	South Africa
SABS	South African Bureau of Standards
SANS	South African National Standards
ssDNA	Single-stranded Deoxyribonucleic acid
STEC	Shiga Toxin-producing *Escherichia coli*
TB	Tuberculosis
TSB-broth	Tryptic Soy Broth
TTP	Thrombocytopenic Purpura
USA	United States of America
VEPAC	Variance Estimation and Precision Analysis Calculation
VRBG	Violet Red Bile Dextrose Agar
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate agar

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CHAPTER 1

INTRODUCTION

Fresh produce consumption is increasing worldwide as consumers are becoming more health-conscious (Leon *et al.*, 2009). In 2013, the South African food-based guideline advised that up to five portions of fruit and vegetables should be consumed a day. Fresh produce is important to human health. A balanced diet prevents chronic diseases and enhances the quality of life (Skinner, 2008a). With the increase of fresh produce production and consumption, foodborne-related outbreaks linked to fresh produce are also increasing (Herman *et al.*, 2015).

Over the last two decades, a general increase in fresh produce-related outbreaks has been observed globally (Herman *et al.*, 2015). In South Africa, there is limited information available on food related outbreaks due to a lack in a surveillance reporting system. However, in the United States the following outbreaks, linked to fresh produce alone, were reported: Three outbreaks occurred during 2017 and 2018 all linked to *E. coli* O157:H7 contaminated Romaine lettuce that affected almost 300 people (CDC, 2017; CDC, 2018a; CDC, 2018b). In 2016, Listeriosis, as a result of *Listeria monocytogenes* contamination traced back to packaged salads (CDC, 2016); A *Salmonella* outbreak was related to contaminated cucumbers in 2015 (Zuraw, 2015); Shiga Toxin-producing *Escherichia coli* was isolated from organic spinach and spring mix (CDC, 2012) and another Romaine lettuce linked outbreak occurred as a result of *Escherichia coli* O157:H7 in 2011 (CDC, 2011). Furthermore, during the years 1973 – 2012, 120 outbreaks were associated with lettuce, nine outbreaks associated with cabbage and five outbreaks associated with spinach (Herman *et al.*, 2015). Herman *et al.* (2015) reported that during the study period there were 20 003 associated illnesses, 1 030 hospitalizations and 19 deaths reported. Shiga Toxin-producing *Escherichia coli* was the cause of 18% of the outbreaks followed by *Salmonella* (11%). Overall leafy green vegetables were the cause of a larger number of outbreaks in comparison with other food groups.

The Food and Agricultural Organization (FAO) and the World Health Organization (WHO) are in agreement that leafy green vegetables are a high-risk product in terms of the microbiological safety and, therefore, prioritised as a level 1 priority. (FAO & WHO, 2008). Leafy green vegetables include lettuce, cabbage, spinach and fresh herbs. The reason for this statement is because of the diverse and complex ways the crops are grown and processed. Post-harvest handling including packing, transporting and storage also gives opportunity for the amplification of foodborne pathogens. Another major reason is because of the substantial number of illnesses that could be traced back to large outbreaks associated with fresh produce. Tomatoes are consumed very frequently (14% of total amount of vegetables) in South Africa (Kassier & Van der Walt, 2000) (STATSSA, 2002) and are prioritised as level 2 based on the risk for microbiological contamination (FAO & WHO, 2008).

Water used for irrigation purposes is a major vehicle for the introduction of foodborne pathogens to fresh produce (Ailes *et al.*, 2008). Runoff from animal production, poor sanitation, sewage and informal settlements near rivers are major contributors to pathogens in surface water (Ashton, 2007). Several studies reported that the microbiological quality of the rivers in the Western Cape is not acceptable for irrigational purposes with a high risk of transferring the pathogens to the fresh produce (Paulse *et al.*, 2012; Van Blommestein, 2012; Romanis, 2013; Lamprecht *et al.*, 2014). The major pathogens associated with water and fresh produce are: pathogenic *Escherichia coli* including Shiga toxin-producing *Escherichia coli, Salmonella* spp*., Listeria monocytogenes, Staphylococcus aureus, Clostridium botulinum, Shigella, Campylobacter spp,* and *Yersinia enterococcus* (Garrett *et al.*, 2003; De Waal & Bhuiya, 2007; Denis *et al.*, 2016; Yeni *et al.*, 2016). Other environmental conditions that contribute to the contamination of fresh produce before harvest include faecal contamination of wild and domestic animals, contaminated soil, contaminated pesticides, growth hormones and liquid fertilisers that increase antibiotic resistance (Olaimat & Holley, 2012).

Antibiotic resistance dissemination in the environment is a worldwide concern, especially Extended Spectrum β-Lactamase (ESBL) producing *Enterobacteriaceae* that is resistant to 3rd and 4th generation Cephalosporins which are critical agents. These agents are often used as the last resort agent for the treatment of critical infections caused by gram-negative bacteria (Cantón *et al.*, 2008). The WHO advisory group on integrated surveillance of antimicrobial resistance, prioritised critically important antimicrobials for human medicine. According to this report there are four classes of antimicrobials that are classified as high priority because (i) there is a high number of people affected by the infection in need for the specific antimicrobial agent, (ii) the frequency of the use of the antimicrobial is high, and (iii) the transmission of the pathogens is from environmental sources, increasing the prevalence of infection. The 3rd and 4th generation cephalosporins (beta-lactam antibiotics) are included in these critically important antimicrobial agents since it is the most important agents for the treatment of ESBL producing *Enterobacteriaceae* (WHO, 2011). ESBL producing *Enterobacteriaceae* is a group of bacteria that produce an enzyme (β-lactamase) that chemically inactivate beta-lactam antibiotics, causing the application of the agent to a vulnerable patient to be ineffective (Blaak *et al.*, 2015). This implies that the last resort antibiotic may not be effective against serious infections caused by gram-negative bacteria (Falagas & Karageorgopoulos, 2009). Some of the typical ESBL producing *Enterobacteriaceae* include *Citrobacter* spp., *Enterobacter* spp*., Escherichia coli, Klebsiella spp*., *Kluyvera, Serratia* and *Rahnella* species (Van Hoek *et al*., 2015).

ESBL producing *Enterobacteriaceae* are commonly found in soil or contaminated surface or irrigation water (Van Hoek *et al.*, 2015). Bacteria can survive in soil for long periods of time depending on the soil composition, temperature and moisture (Honjoh *et al.*, 2014). Good conditions for bacterial survival are nutrient-rich soil and high moisture. *Salmonella serviva* can survive up to 200 days in soil even at low temperatures (Honjoh *et al.*, 2014). A 2012 study also reported that there is a significant carry-over effect of pathogens from contaminated irrigation water to fresh produce (Van

Blommestein, 2012). Kroupitski *et al.* (2011) confirmed *Salmonella* spp. will aggregate near the stomata of the lettuce when the pathogen ends up on the crops via contaminated irrigation water.

Other contamination sources also play a role in the dissemination of pathogens to fresh produce. Postharvest contamination factors include human handling, harvesting equipment, transport containers, wash & rinse water, sorting & cutting equipment, storage and transportation (Harris *et al.*, 2003). Heads of lettuce are often harvested by hand and packed in the field (Matthews, 2013). This increases the risk of spread of pathogens from the handler's hands to the crops. Jimenez *et al.* (2007) reported that the transfer of *Salmonella* was high between the volunteer's hands and green bell peppers during a bidirectional transfer study. The growth of the contaminated product may be enhanced by increased temperatures and open transport. The *E. coli* O157:H7 counts increased 11 times in 4 hours at 28˚C (Brandl, 2008). The latex, the milky fluid of the plant, leaks out of fresh cut produce and creates a nutrient and moisture rich environment that facilitates rapid multiplication (Brandl, 2008). The risk for the survival and growth of foodborne pathogens are, therefore, increased and amplified by post-harvest processes.

All consumers are susceptible to foodborne related illnesses caused by the consumption of contaminated food. The major foodborne pathogens isolated from fresh produce includes *Salmonella* spp, pathogenic *E. coli* and *Listeria monocytogenes*. Although all consumers can be affected by foodborne illnesses, the immunocompromised individual carries the highest risk for infection. Immunocompromised consumers include HIV positive (11.2% prevalence rate in South Africa) (STATSSA, 2015) and TB sufferers (South Africa is one of the top three world countries with the worst TB epidemics (WHO, 2014)). Other consumers that may have weakened or impaired immune systems are children under the age of 10 years (20.8% of the total South African population), the elderly above the age of 65 years (8% of the South African population) and pregnant woman (STATSSA, 2015). According to the American Produce for Better Health Foundation (2015) the elderly (aged 65 + years) is the age group that has the largest vegetable consumption of 71% which is 27% more than the average age group consumption. Therefore, the contaminated fresh produce leaves a large percentage of the South African population at risk for foodborne related infections. There is also a significant correlation between immunocompromised individuals and individuals living in informal settlements (Ncayiyana *et al.*, 2016).

According to a study completed in 2008 in Durban, South Africa, there is a significant correlation between informal trading and high poverty areas (Skinner, 2008b). Limited statistics are available regarding informal trading before 1994 because it was banned before then. More recent statistics have shown that the informal market is a big part of the fresh produce industry. The total South African fresh produce consumption in 1999 was 3 600 000 tons, of which 55% was sold in fresh produce markets. The rest was either sold in direct sales to the trade (8%), direct exports (6%), direct sales to processors (11%) or was held back for farmer consumption (20%). Thus, the informal market (fresh produce markets) is responsible for the highest distribution of fresh produce (Kassier & Van der Walt, 2000). In 2012, the province with the highest fresh produce production in South

Africa was Gauteng (45.61%) and the Western Cape (53.52%). Furthermore, from 2002 – 2011, the Western Cape became the province with the highest market share for lettuce exports (53.53%) with Gauteng in second with 45.61% (DAFF, 2015). This highlights the importance of knowing the food safety status of fresh produce in the Western Cape both for the fresh produce market and for a big part of the South African population that has a high risk for foodborne diseases.

The safety of fresh produce can be determined in many ways. Indicator organisms can give an indication of the handling practices used during production and post-harvest handling of the crop (Forsythe, 2010). *E. coli* is a human enteric organism and is used as an indicator because of the direct correlation between the presence of *E. coli* and poor handling practices (Busta *et al.*, 2003). The guidelines for environmental health officers on the interpretation of microbiological analysis data of food from the Department of Health, South Africa, proposes microbiological specifications for fresh produce (DoH, 2000). The non-compulsory guideline for fresh produce proposes a zero tolerance for *E. coli* (0 cfu.g^{-1}). Another frequently used indicator system is the enumeration of coliforms. The guidelines propose a limit of less than 200 cfu.g^{-1} for acceptable microbiological levels (DoH, 2000). Indicators alone is not sufficient to comment on the safety status of fresh produce. It is important to test for high-risk foodborne pathogens as well. The tolerance for the presence of *Salmonella* spp. is zero in 25 g. The international alliance for street vending organisations has warned that street vendors have no best practice policies to control the food safety risk (Skinner, 2008a). This poses a risk to consumers because each street vendor owner handles the fresh produce based on the individual's knowledge and there is no regulatory control that monitors the safety of the produce that is sold at street vendors in South Africa.

Previous studies reported on the microbiological safety of fresh produce in Gauteng. Van Dyk *et al.* (2016) reported coliform levels between 4.2 to 6.2 log cfu.g^{-1} on tomatoes during an cross-sectional study on tomatoes throughout the supply chain. Jongman & Korsten (2017) assessed the microbiological safety of lettuce, cabbage and spinach in different production systems. There were no pathogens detected on the fresh produce, however, the presence of *E. coli* was detected. The average coliforms present on the fresh produce were 4 log cfu.g^{-1}. An exploratory study, by Du Plessis *et al.* (2017), into the microbiological safety of spinach and cabbage purchased from street vendors and formal retailers in Johannesburg reported that the coliform counts exceeded the microbiological limit advised by the Department of Health by far. The presence of *Salmonella* spp. and *L. monocytogenes* was detected in 7.2% and 5% of the total samples, respectively. The presence of *E. coli* was also detected. However, to the best knowledge of this author no information is currently available from the Western Cape.

The aim of this study was to determine the status of the microbiological safety of selected fresh produce (lettuce, spinach, cabbage, tomatoes, green peppers and green beans) sold at informal retailers at selected areas in Cape Town Metropolitan area in South Africa. This was achieved by enumerating indicator populations present on the selected fresh produce, and screening for high-risk pathogens frequently associated with fresh produce including *Salmonella* spp., Shiga-

toxin producing *E. coli* and *Listeria monocytogenes*. The presence and prevalence of Extended Spectrum β-Lactamase (ESBL) producing *Enterobacteriaceae*, was also evaluated. This was achieved by initially screening for ESBL *producers* using ESBL ChromID (Biomerieux) agar and confirming ESBL status phenotypically with antibiotic disc diffusion test and by PCR.

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CHAPTER 2

LITERATURE REVIEW

2.1. Fresh produce consumption

Fresh produce consumption is increasing globally as consumers become more health-conscious (Leon *et al.*, 2009). In the last two decades, the global fresh produce consumption has increased by 10% which leads to a need for improvement in the food safety systems for pre- and post-harvest systems (Kaufman *et al*., 2000). In contrast, the fresh produce consumption in South Africa has decreased by 15% since 1994 (Ronquest-Ross *et al.*, 2015). This, however, could be due to the recent droughts South Africa has experienced in the last decade and the rise in overall food prices by 75% (World Bank, 2008). Never the less, fresh produce that are harvested and sold must still be safe for consumption. This, however, puts an enormous stress on the food industry to harvest a quality product that is both safe for consumption and free of foodborne pathogens (Castro-Ibáñez *et al*., 2015).

There is limited information available about the consumption of fresh produce in South Africa since 1994. However, in 1999, the total South African fresh produce consumption was 3 600 000 tons which 55% was sold in informal fresh produce markets. The rest was either sold in direct sales to the trade (8%), direct exports (6%), direct sales to processors (11%) or was held back for farmer consumption (20%). The informal market (fresh produce markets) is therefore responsible for the highest distribution of fresh produce (Kassier & Walt, 2000). The main vegetables consumed in South Africa are: potatoes (39%), tomatoes (14%), onions (11%), green mealies (8%), cabbage (6%), pumpkins (8%), carrots (3%) and other vegetables (11%) as reported by Kassier and Van der Walt (2000)

2.1.1. Fresh produce production and economic impact in South Africa

South Africa is the third largest global supplier of fruit and vegetables to Europe (Ndiame & Jaffe, 2005). Out of all the vegetables that are grown in South Africa, 73% of the vegetables are exported (Ndiame & Jaffe, 2005). The South Africa cabbage exports to Africa include Lesotho (47% of SA grown cabbage), Botswana (31%), Swaziland (8%), Namibia (6%) and others including Angola, Mozambique, Zambia, Gabon and Congo (Kassier & Van der Walt, 2000). The main export countries of lettuce are Angola (57.3% of South African lettuce export share), Congo (16%) and Mozambique (11.4%) (DAFF, 2015).

Cape Town held the third largest market share of fresh produce (13.3%) with Pretoria that held 15.5% and Johannesburg 31.7% in 1996 (Rathogwa *et al.*, 1996). In 2008, the main sources for fresh produce in South Africa mainly originated from Gauteng (60.21%) and the Western Cape (13.31%) making the Gauteng the province with the largest export economic impact (Chikazunga *et*

al., 2008). However, from 2002 – 2011 the Western Cape became the province with the highest market share for lettuce exports (53.53%) with Gauteng in second with 45.61% (DAFF, 2015). Furthermore, out of all the districts in the Western Cape, the city of Cape Town held the largest lettuce exports (97.01%) in comparison with other lettuce producing districts such as Overberg (2.99%) for example. Therefore, making Cape Town almost the sole supplier of lettuce in the Western Cape.

The fresh produce market plays an important role in the South African agricultural industry and economy. Any bad publicity as a cause of microbiological unsafe fresh produce or foodborne outbreaks can lead to economic losses. The informal market has a large impact on the South African economy since 1994 but is not recognised by economists (Skinner, 2008). The reasons for this will be discussed in section 2.1.2.

2.1.2. Informal vendor trading in South Africa

Little statistics are available of the number of traders currently trading informally. Informal trading was largely banned before 1994. However, a study has shown that between 16 and 20% of the South African labour force was working informally. Recalculated household survey data were compared to labour force surveys that indicated on average, over the time period of 2001 to 2007, half of those individuals that indicated that they were working informally, were involved in street vendor trade. In 1997, in the Labour Force survey, 19 301 informal street vendors were found in the Durban Metropolitan area which half of those were found to trade with food (Skinner, 2008). Unfortunately, it is difficult to determine exact numbers of informal traders in South Africa. There is, however, enough evidence that informal food trading plays a major role in the South African food market and accounts for an estimated 10% of all fresh produce trading (Skinner, 2008).

Skinner (2008) reported that there is a significant correlation between informal trading and high poverty areas in South Africa. In the Western Cape, 16.6% of households reported living in an informal dwelling (STATSSA, 2016). Overall in South Africa, 13.0% of the population is living in informal dwellings (STATSSA, 2016). This is a significant part of the South African population that is probably purchasing their fresh produce from informal vendors. There is also a significant correlation between individuals living in informal settlements and individuals suffering from immunocompromised health issues (Ncayiyana *et al.*, 2016). Immunocompromised individuals stand a higher risk of foodborne infections caused be foodborne pathogens. These individuals are identified and discussed in section 2.1.3.2.

2.1.3. Microbiological safety of fresh produce

According to the research done by the FAO & WHO (2008), leafy greens was ranked as level 1 because of the concern for microbiological contamination. The classification system used to rank the different food types include: the frequency and severity of the microbiological contamination, the size of the production of the crop, and the potential for export and economic impact of the food group.

However, the main reason for the risk ranking of fresh produce is because of the diverse and complex ways these crops are grown and processed. According to the FAO and WHO, leafy green vegetables, that is classified as high risk (level 1), include spinach, lettuce, salad leaves and fresh herbs. The level 2 priority food groups include berries, green onions, melons, sprouted seeds and tomatoes. Level 3 priority food types include carrots, cucumber, almonds, baby corn, sesame seeds, onions and garlic. These are all food products that are mostly consumed raw. The risk to the consumer is therefore higher because there is no heat or processing step to reduce the initial microbial load on the product.

2.1.3.1. Recent outbreaks associated with fresh produce

Over the last two decades, a general increase in fresh produce-related outbreaks in the United States was reported. The most recent outbreaks occurred as a result of *E. coli* O157:H7 contamination traced back to Romaine lettuce that affected almost 300 people in more than 30 states in America. These outbreaks occurred in 2017 and two outbreaks in 2018 (CDC, 2017; CDC, 2018a; CDC, 2018b). In 2016, Listeriosis as a result of *Listeria monocytogenes* contamination was traced back to packaged salads (CDC, 2016). A *Salmonella* outbreak was traced to contaminated cucumbers in 2015 (Zuraw, 2015). Shiga Toxin-producing *Escherichia coli* (STEC) was isolated from organic spinach & spring mix (CDC, 2012) and a Romaine lettuce outbreak as a result from *Escherichia coli* O157:H7 was reported in 2011 (CDC, 2011). Furthermore, during the years 1973 – 2012, 120 outbreaks were associated with lettuce, 14 outbreaks associated with unspecified leafy greens, nine outbreaks associated with cabbage and five outbreaks associated with spinach (Herman *et al.*, 2015). Herman *et al.* (2015) reported that during the study period there were 20 003 associated illnesses, 1030 hospitalizations and 19 deaths reported. STEC was the cause of 18% of the outbreaks followed by *Salmonella* (11%). Overall leafy green vegetables were the cause of a larger number of outbreaks in comparison with other foods (Herman *et al.*, 2015).

2.1.3.2. Individuals that are at risk as a cause of consumption of possible contaminated fresh produce

The individuals affected by contaminated fresh produce will be dependent on the specific pathogen that caused the foodborne outbreak. As mentioned in section 2.1.3.1 *Salmonella*, Shiga toxin producing *Escherichia coli* and *Listeria monocytogenes* are some of the major foodborne pathogens that caused foodborne outbreaks related to fresh produce. Salmonellosis (as a cause of *Salmonella* infection) and pathogenic *E. coli* intake affects all who come in contact with the respective foodborne pathogen and it's onset symptoms could be lethal if not treated. However, *Listeria monocytogenes* mostly only affect pregnant woman and individuals that are immunocompromised, including children under the age of 10 years (20.8% of the total South African population) and the elderly above the age of 65 years (8% of the South African population) (STATSSA, 2016; Forsythe, 2010). Other immunocompromised individuals include individuals with HIV (11.2% prevalence rate in South

Africa) (STATSSA, 2016) and a significant number of TB sufferers, as South Africa is one of the top three world countries with the worst TB epidemics (WHO, 2014). Considering the South African statistics, about 30% of the population is vulnerable to foodborne illnesses leaving a large part of the population vulnerable to foodborne diseases as a result of contaminated fresh produce.

2.2. Prevalence of foodborne pathogens associated with fresh produce

The major pathogens associated with water and vegetables that have the ability to survive are pathogenic *Escherichia coli* including Shiga toxin-producing *Escherichia coli, Salmonella* spp*., Listeria monocytogenes, Staphylococcus aureus, Clostridium botulinum, Shigella, Campylobacter* spp*, Yersinia enterococcus* and *Bacillus cereus* (Beuchat, 2002; Garrett *et al.*, 2003; De Waal & Bhuiya, 2007; Denis *et al.*, 2016; Yeni *et al.*, 2016). Extended-Spectrum Beta-Lactamase producing *Enterobacteriaceae* (ESBL) has recently also become a concern due to its resistance against third generation antibiotics and its prevalence and rapid spread in the environment (Denis *et al.*, 2016).

2.2.1. Survival and growth

Fresh produce is a natural habitat of bacteria because of its high moisture content and nutrient content. It also contains natural openings including the stomata and lenticels which make it ideal for the initial attachment and survival of pathogens (Forsythe, 2010; Yeni *et al.*, 2016). Some fresh produce, for example tomatoes or green peppers, may have shells, a waxy cover or a low pH that prevents the survival of pathogens. The low pH of fully ripe tomatoes ranges from pH 3.9 – 4.4 that will prevent or reduce the growth speed of pathogens (Beuchat, 2002). In contrast, the optimum pH for yeast and moulds are in the range between pH 2.2 – 5.0. These conditions give yeast and moulds an advantage above bacteria and create a competitive environment. Fresh produce, especially fruit, may spoil because of the competitive growth of yeasts and moulds (Beuchat, 2002). Leafy vegetables have medium acidity with a pH that ranges between pH 4.0 – 6.0 that makes it a comfortable environment for the growth of bacteria (Forsythe, 2010). However, fruit and vegetable with a pH lower than 4.0, generally do not support the growth of pathogenic bacteria (Beuchat, 2002).

The spoilage of fresh produce is often caused by environmental bacteria including *Pseudomonas*, *Xanthomonas*, *Erwinia*, *Bacillus*, *Clostridium* and some strains of yeasts and moulds (Lund & Brocklehurst, 1981; Brackett, 1993;). Bennik & Van Overbreek (1999) reported that some naturally occurring bacteria can have a bacteriocinogenic effect and prevent the growth of pathogenic bacteria, for example, *Pediococcus* and *Enterococcus* that prevents the growth of *Listeria monocytogenes* (Bennik & Van Overbreek, 1999). Other studies reported that bacterial soft rot can increase the survival and growth of foodborne pathogens (Wells & Butterfield, 1997). Wells & Butterfields (1997) reported that 41% of all raw fruit and vegetables sampled (n=401) in their study that was affected by bacterial soft rot were positive for presumptive colonies of *Salmonella.*

Therefore, vegetables that indicated some spoilage due to spoilage bacteria increased the risk of the growth of pathogenic bacteria if present.

Bacteria have the ability to secrete extracellular polymeric substances (EPS) that influences the bacteria's adhesion properties in natural environments. The EPS layer consist of exopolysaccharides and serves as a boundary to keep in nutrients and prevent antibiotics or some sanitisers to cause damage to the cells (Vu *et al.*, 2009). EPS layers are a key component in the formation in biofilm which is another survival mechanism for pathogens on the surface of fresh produce (Vu *et al.*, 2009).

2.2.2. Highly prevalent microorganisms in fresh produce

2.2.2.1. Gram-positive pathogens

Listeria monocytogenes

Listeria monocytogenes is a gram-positive, non-spore forming, facultative anaerobe, motile bacteria. *Listeria* was described for the first time in 1924 by E.G.D. Murry and was only identified later as a foodborne pathogen (Bowers & Elston, 1958). The *Listeria* genus is classified into six species including *L. monocytogenes, L. innocua, L. welshimeri, L. grayi, L. seeligeri* and *L. ivanovii. L. monocytogenes* is the only species that is classified as a human pathogen but recently *L. ivanovii* was also documented to have caused listeriosis in humans (Forsythe, 2010; Nyarko & Donnelly, 2015). *Listeria* is classified into four lineages. Lineage I is mainly associated with human Listeriosis whereas Linage II is mainly recovered from environmental isolates including fresh produce (Milillo & Wiedmann, 2009). *L. monocytogenes* infection is a cause of lethal infections to infants, the elderly and immunocompromised individuals such as HIV and TB sufferers but especially pregnant women and their young. The mortality rate of *Listeria monocytogenes* is very high in comparison to other foodborne pathogens although fewer individuals are infected. During pregnancy, the infection can be transferred from the mother through the placenta, resulting in stillborn or premature birth (Yeni *et al.*, 2016). *Listeria monocytogenes* is commonly found in the environment and can contaminate fresh produce via the soil, water, animal faeces, sewage, insects or decaying vegetation (Jay *et al.*, 2005). Moreover, this pathogen can tolerate high salt concentrations and is comfortable in a temperature range from 0˚C to 37˚C. Thus, *Listeria monocytogenes* can survive at refrigeration temperatures (Forsythe, 2010).

Staphylococcus aureus

Staphylococcus aureus is a rod-shaped, facultative anaerobic, non-motile and non- spore-forming bacteria that produces staphylococcal enterotoxins causing food poisoning (Forsythe, 2010). Symptoms caused by the staphylococcal enterotoxin include nausea, abdominal pain, vomiting and diarrhoea (Forsythe, 2010). Human and animals are the primary reservoirs of this foodborne

pathogen although it is also found in dust and the air. *Staphylococcus aureus* cross-contamination mainly occurs from infected humans by sneezing, coughing or from contaminated hands (Behling *et al.*, 2010). Human handling is the main cause of *Staphylococcus* contamination.

2.2.2.2. Enterobacteriaceae

Salmonella spp.

Salmonella is gram-negative, facultative anaerobic, non-spore forming rods. Salmonellosis is caused by the infection by either a nontyphoidal strain which has milder symptoms including diarrhoea, vomiting and abdominal pain however the typhoidal strain has more severe health implications including a severe fever (Forsythe, 2010). The incubation period of *Salmonella* is 12-72 hours depending on the individual's health. Individuals that suffer as a cause of *Salmonella* infections can experience symptoms that last up from 4-7 days (Forsythe, 2010). Food that is commonly associated with *Salmonella* contamination are: contaminated eggs, poultry, meat, unpasteurized milk, cheese, spices and nuts, and contaminated raw fruit and vegetables. Separating raw foods from ready to eat foods to reduce cross-contamination is important as well as the maintenance of the cold chain during transportation. *Salmonella* is very common in the natural environment including the soil and water. This organisms' primary reservoirs are the gut of humans and animals from where it is spread through the use of animal manure as fertiliser. Fresh produce can be contaminated by *Salmonella* during pre-harvest by contaminated irrigation water, soil, insects or via animal manure. Cross-contamination during post-harvest processing activities is also a possibility from contaminated equipment, surfaces and handlers (Yeni *et al.*, 2016).

Escherichia coli

Escherichia coli is a gram-negative, non-spore forming, facultative anaerobe, short rod-shaped bacteria that belong to the genus *Enterobacteriaceae* (Forsythe, 2010). It is part of the normal gut bacteria in humans an in animals especially ruminants (Yeni *et al*., 2016). *E. coli* is divided into six pathogenic groups based on their symptoms and mechanisms of pathogenicity: Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC) also known as Shiga Toxin-producing *E. coli* (STEC), Enteroaggregative *E. coli* (EAggEC), and Diffusely Adherent *E. coli* (DAEC) (Forsythe, 2010). STEC is by far the *E. coli* group that causes the most severe symptoms and a major concern for the health of an infected individual (Forsythe, 2010). Symptoms include bloody diarrhoea, thrombocytopenic purpura (TTP), haemorrhagic colitis and Haemolytic-uremic syndrome (HUS) (Forsythe, 2010). The infective dose for STEC is about 10 cells or less to cause a severe illness, therefore making it a high-risk organism. The individuals that are the most at risk for EHEC, EPEC or EAggEC infection are infants/ children under the age of six and the elderly. In some cases, HUS may develop chronic kidney disease or even kidney failure (Weiss & Schmids, 2011). STEC is mostly found in environments where livestock

is present and is isolated mostly from faeces (Yeni *et al*., 2016). Some of the routes pathogens are spread is through faecal matter that is transferred to fresh produce through contaminated irrigation water or soil. During preparation, post to harvest, cross-contamination is also a vehicle of cross-contamination via the use of contaminated equipment, surfaces or the hands of food handlers (Yeni *et al*., 2016). Finally, the pathogen ends up on the fresh produce which is consumed raw, therefore cooking is not an option to reduce the risk or the elimination of pathogenic *E. coli*.

Shigella spp.

Shigella is a nonmotile, rod-shaped, non-spore forming bacteria which causes severe illnesses ranging from diarrhoea to enterotoxin or shigatoxin related HUS. Infectious species include *S. sonnei, S. boydii, S. flexneri* or *S. dysenteriae* (Forsythe, 2010). The primary source of *Shigella* is infected humans and it can be spread via water or insects that was contaminated by human faeces (Forsythe, 2010). Rivers near informal settlements that do not have proper sanitation infrastructure is high risk. Because humans are the primary reservoir, contamination via handling during post-harvest is a big risk if the handler is infected by *Shigella* (Jay *et al.*, 2005). These pathogens can easily survive on fresh produce because *Shigella* is hardened to survive in conditions where the surface is dry, the temperature is low or the pH is low (Forsythe, 2010).

Enterobacter spp.

Enterobacter is a gram-negative, facultative anaerobic, rod-shaped and non-spore-forming bacteria. Two species of clinical importance is *E. aerogenes* and *E. cloacae.* These opportunistic pathogens have emerged as nosocomial pathogens in pathogens in need of ventilator equipment in the intensive care units in hospitals (Davin-Regli & Pagès, 2015). *Enterobacter* is commonly found in plant vegetation and soil and is therefore often associated with fresh produce (Forsythe, 2010).

2.2.2.3. Antibiotic resistance

In the pre-antibiotic era the mortality rate for simple infections was fearfully high. Since the first antibiotic was discovered in 1928 by Alexander Fleming the development of antibiotics increased rapidly. Since the discovery of penicillin, the birth of the first antibiotic class, 21 different classes of antibiotics were discovered and developed further (McArthur *rt al*., 2013). The discovery of antibiotics opened a new era where the fight against infections caused by bacteria can be overcome (Van Boeckel *et al.*, 2014). The global consumption of antibiotics increased by 35% in a decade's time between the years 2000 and 2010 whereas the global population increase was only 31%. This shows a general increase in the use of antibiotics regardless of the growth in population (Van Boeckel *et al.*, 2014). However, with the rapid incline in antibiotics use, an increase in antibiotic resistance is also a reality. Bacteria have the natural ability to adapt to environmental conditions to fight for survival even in stressful and harsh conditions (Wellington *et al.*, 2013). The short lifetime and rapid growth rate of bacteria make them more adaptable to undergo genetic changes to survive

the treatment of antibiotics. Bacteria develop resistance against antibiotic treatment by exchanging beneficial genes from one to another by a mechanism called horizontal gene transfer (Wellington *et al.*, 2013). However, this is not the only mechanism for the spread of genes causing resistance in bacteria.

Extended-spectrum Beta-lactamases (ESBLs) was first described in the 1980's as a major threat amongst the multi-drug resistant bacterial isolated in the nosocomial environment (Cantón *et al.*, 2008). ESBLs were first recognised in Europe predominately among *Enterobacteriaceae* but has since then increasingly been described worldwide (Paterson & Bonomo, 2005). In 1983, in Germany, the first *Escherichia coli* was recovered from a patient in the intensive care unit (ICU). The *E. coli* strain had an abnormal resistance against Cefotaxime and Ceftazidime which was transferable by conjugation to *E. coli* (Knothe *et al*., 1983). This isolate contained a variant of the classic SHV-1 enzyme that was later named SHV-2 (Knothe *et al*., 1983). One year later, 1984, *Klebsiella pneumonia* with an identical phenotype was detected in France except that that these isolates, detected in various hospitals, now had a variant of the broad spectrum TEM-2 β-lactamase. Just like the SHV-2, the TEM-3 enzyme was also transferable by conjugation (Sirot *et al.,* 1987). Table 2.1 is a summary of the spread of ESBLs in the first decade by the year the isolate was first reported. During the year of the 1970's, the bla_{SHV} and bla_{TEM} gene was widespread as the genes of concern. After a widespread pandemic of CTX-M producing organisms in the 2000's, it was the new gene of concern that causes the resistance of ESBL *Enterobacteriaceae* particularly in the organism *E. coli* and *K. pneumonia* (Cantón & Coque, 200; Falagas & Karageorgopoulos, 2009).

ESBL producing *Enterobacteriaceae* is a group of bacteria that have antibiotic resistance against beta-lactam antibiotics (Blaak *et al.*, 2015). Some of the typical ESBL-producing *Enterobacteriaceae* include *Citrobacter* spp., *Enterobacter* spp*., Escherichia coli*, *Klebsiella* spp., *Kluyvera, Serratia* and *Rahnella* species which are found in soil or contaminated surfaces or irrigation water (van Hoek *et al.*, 2015). ESBL producing *Enterobacteriaceae* has recently been isolated from fresh produce. Kim *et al*. (2015) have claimed to be the first to report on the presence of ESBL producing *Escherichia coli* and *Klebsiella pneumonia* in ready-to-eat (RTE) vegetables. This study, based in South Korea, reported a 10.1% (n=189) prevalence of ESBL producing *Escherichia coli* and *Klebsiella pneumonia* in RTE vegetables (Kim *et al*., 2015). All the isolates were resistant to Cefotaxime, and many of the ESBL producers was resistant to gentamicin, trimethoprim/sulfamethoxazole, and ciprofloxacin as well (Kim *et al*., 2015). A similar study was conducted in the Netherlands and found a prevalence rate of 5.2% (n=1116) of RTE vegetables carrying ESBL resistant organisms (Van Hoek *et al.,* 2015). Both these studies expressed their concern over the spread of the antimicrobial resistant bacteria and ESBL genes to humans by consuming contaminated RTE vegetables.

Table 2.1 A summary of the emergence of Extended-Spectrum β- Lactamase families in Europe adapted from Jacoby & Medeiros (1991)

β-lactamase	Species	Country of origin	Year of first report
TEM-3	*K. pneumonia*	France	1984
TEM-4	*E. coli*	France	1986
TEM-6	*E. coli*	Germany	1987
TEM-9	*K. pneumonia*	England	1987
TEM-10	*K. pneumonia*	United States	1989
TEM-11	*K. pneumonia*	Belgium	1989
TEM-20	*K. pneumonia*	Tunisia	1990

Factors influencing antibiotic resistance

Water is an excellent mechanism for the spread and contribution to the rise of antibiotic resistant organisms (Lupo *et al.*, 2012). Bacteria are transported in different ways to urban, industrial and agricultural waste and are collected and mixed at water collecting points such as dams and rivers. This is where clinically important bacteria are mixed with environmental species that contains intrinsic antibiotic resistance genes. The concern is that these resistant genes are transferred from initially clinical isolates to environmental organisms. There are many factors contributing to antibiotic resistance in the environment. Antibiotic misuse in the clinical sector is not the only factor. Antibiotics is used in the agricultural environment as growth promoters in feed, for feed efficiency and for animal disease treatment (Sarmah *et al.*, 2006). The antibiotic agent is fed to the animal either via feed, water, by injection, paste, orally, pour on or bolus. However, the antibiotics that are applied orally has the greatest effect on antibiotic resistance (Sarmah *et al.*, 2006). A few studies have shown that 30-90%, depending on the antibiotic, is not absorbed by the gut of the animal when administered and is being excreted (Elmund *et al.*, 1971; Alcock *et al.*, 1999; Sarmah *et al.*, 2006). However, Marti *et al.* (2013) reported that there were still numerous resistant genes found in unmanured soil in comparison to manured soil during the study conducted in 2012 suggesting that manure is not the only vehicle for the distribution of resistance genes.

Antibiotic use

Rising antibiotic resistance is a worldwide concern. *Enterobacteriaceae* that is resistant to 3rd generation Cephalosporins, that are critical agents against gram-negative bacteria, is however a major health threat (Cantón *et al.*, 2008). This raises a health concern due to the difficulty to treat infections caused by ESBL-producing *Enterobacteriaceae* as Beta-lactam antibiotics are often used

as a so-called “last resort” antibiotic (Blaak *et al.*, 2015). The antibiotics mostly used in the agricultural sector varies from country to country. The global use of antibiotics in the agricultural sector that is used most includes the following: β-lactams, Sulphonamides, Tetracyclines and Aminoglycosides (Sarmah *et al.*, 2006). The antibiotics important for human health are ranked by WHO into four categories based on the importance of the antibiotic and is summarised in Table 2.2 (WHO, 2012).

Table 2.2 The World Health Organisation listings of the most important antibiotics for human health (WHO, 2012)

Critically important	Highly important	Important	Unclassified
Aminoglycosides	Amdinopenicillins	Aminocyclitols	Ionophores
Carbapenems and other penems	Amphenicols	Cyclic polypeptides	Bambermycins
Cephalosporins (3rd and 4th generation)	Cephalosporins (1st and 2nd generation)	Nitrofurantoins	Carbadox
Fluoro- and other quinolones	Steroid antibacterials	Nitroimidazoles	
Glycopeptides	Streptogramins		
Macrolides and ketolides	Sulfonamides		
Monobactams	Sulfones		
Oxazolidinones	Tetracyclines		
Penicillins (natural aminopenicillins and antipseudomonal)	Penicillins (anti-staphylococcal)		

2.2.2.3.1. β-lactamase antimicrobials

The β-lactam antibiotic group is a highly integrated and broad antibiotic group. The β-lactamases antimicrobials can be subdivided into groups according to their functional similarities (Bush-Jacoby Medeiros classification) or classified into classes based on their molecular structure (Amber classification). Often an antibiotic is classified in one group (Bush-Jacoby group) (Bush & Jacoby, 2010) but is classified in more than one Amber class (McArthur *et al.*, 2013). Table 2.3 shows a brief summary of the β-lactamases classification. β-lactamases are classified into three groups according to the Bush-Jacoby classification and into five subclasses according to the Amber classification. The enzyme, β-lactamases, can have an effect on both gram-positive and gram-negative microorganisms (Coyle, 2015). In gram-negative organisms, the β-lactam antimicrobials enter the bacterial cell through the porin proteins located on the outside of the cell. The β-lactam antimicrobials move through the channels and bind to the penicillin proteins. Penicillin-binding proteins (PBPs) are responsible for cell layer synthesis. With the binding of the β-lactam molecules, the PBPs are blocked and cannot carry out their function. This causes a weakened or defective cell membrane and leads to cell lysis and death (Ambler, 1980; Coyle, 2015). In gram-positive bacteria β-lactamases as a similar mode of action, except that gram-positive bacteria do not have an outer membrane. The β-

lactam antimicrobials simply just diffuse through the cell wall and then bind to the PBPs causing the same lethal effect than for gram-negative bacteria (Coyle, 2015). Colostin is an excellent antimicrobial agent against ESBL producing organism but is scarcely used because it is reserved as the treatment of advanced infections of ESBL producing *Enterobacteriaceae* (Falagas & Karageorgopoulos, 2009). The classification of β-lactamases antimicrobials is summarised in Table 2.3.

Table 2.3 Classification of β-lactamases (Bush & Jacoby, 2010; Kocsis & Szabó, 2013; McArthur *et al.*, 2013)

Bush-Jacoby Group		Amber classification	Antibiotic	Inhibited by CA*	Representative enzyme
1	Cephalosporinases	C	Cephalosporins	No	AmpC,
2a	Serine β-lactamases	A	Penicillin's	Yes	PC1
2b		A	Penicillin's, early cephalosporins	Variable	TEM, SHV
2c		A	Carbenicillin	Yes	PSE, CARB
2d		D	Cloxacillin	Variable	OXA
2e		A	Extended spectrum cephalosporins	Yes	CepA
2f		A	Carbapenems	Variable	KPC, IMI, SME, NMC
3a	Metallo-β-lactamases	B	Carbapenems	No	MBL

* Clavulanic acid

Cephalosporins

Cloxacillin

Cephalosporins (3rd and 4th generation) is the first choice of antibiotics to treat infections caused by ESBL producing bacteria (Ehlers *et al.*, 2009). Other antibiotics that the medical field are limited to for the treatment of ESBL related infections are Fluoroquinolones and Aminoglycosides.

Cloxacillin (classified as a Cephalosporin) can be hydrolysed by an OXA-type β-lactamases enzyme (Falagas & Karageorgopoulos, 2009). The enzyme, OXA, is named after its mechanism and hydrolysing activities. These enzymes are the most dominant found in *Pseudomonas aeruginosa* but have been isolated from 1-10% *E. coli* isolates according to a study by Livermore (1995) as well. Organisms containing OXA enzyme hydrolyses Cefotaxime, Ceftriaxone, and Aztreonam making the administration of these antibiotics less effective to treat the infection (Toleman *et al., 2003)*.

Similarities have been found in the evolution of the OXA-type β-lactamases enzyme and the TEM and SHV- type ESBL's (Falagas & Karageorgopoulos, 2009). In the past SHV and TEM-type ESBL's was very prominent, however, over the past decade the OXA-type ESBLs became more prevalent and gained more attention together with the dissemination of CTX-M enzymes (Cantón & Coque, 2006). Cloxacillin, however, is still active against the AmpC activity (Philippon *et al.*, 2002).

Cefoxitin

Part of the Cephamycin's antibiotic class. Co-resistance is observed to Cefoxitin in ESBL producing *Enterobacteriaceae* (Falagas & Karageorgopoulos, 2009). This is mainly due to porin loss or a natural expression of AmpC β-lactamases (Falagas & Karageorgopoulos, 2009). Cefoxitin inhibits plasmid-mediated AmpC enzymes (Philippon *et al.*, 2002).

Penicillins

Ampicillin

Ampicillin that is classified under the penicillin antibiotic agents is also the preferred drug to treat infections caused by ESBL producing *Enterobacteriaceae* (Sarmah *et al.*, 2006; Ehlers *et al.*, 2009). This antibiotic is not only for human use but also for the use in disease prevention in livestock (Sarmah *et al.*, 2006). In the UK 1487 tons of Ampicillin was sold in the year 2000 for the use of animal welfare only (Sarmah *et al.*, 2006).

β-lactamase resistance

There are three basic mechanisms bacteria use to fight against β-lactam antibiotics: (i) the possession of an altered penicillin-binding protein (PBP) with low affinity for β-lactams that does not allow the binding of β-lactamase to PBPs and therefore the cell membrane synthesis continues; (ii) efflux pumps that additionally use β-lactams as substrates and (iii) β-lactamases that cleave to the amide bond of the β-lactam ring which thus inactive the antibiotic agent (Pfeifer *et al.*, 2010). Table 2.4 is a summary of the different β-lactamases enzymes causing resistant against β-lactamase antibiotics, classified according to the Amber classification scheme.

Table 2.4 The Amber classification scheme of the Serine-β-lactamases group (adapted from Pfeifer *et al.*, 2010, originally from Ambler, 1980)

β-lactamase-class	β-lactamases	Preferential occurrence	Important phenotypical resistance traits*
A	Broad-spectrum β-lactamases	*Enterobacteriaceae* and nonfermenters	Ampicillin, cephalotin
	ESBL TEM-type		Penicillins, 3rd generation cephalosporins
	ESBL SHV-type		
	ESBL CTX-M-type		
	Carbapenemases (KPC, GES, SME)		All β-lactams
C	AmpC cephamycinases (chromosomal-encoded) [AmpC]	*Enterobacter spp.* *Citrobacter spp.*	Cephamycins (cefoxitin), 3rd generation cephalosporins
D	AmpC cephamycinases (plasmid-encoded) [CMY, DHA, MOX, FOX, ACC]	*Enterobacteriaceae*	Cephamycins (cefoxitin), 3rd generation cephalosporins
	Broad-spectrum β-lactamases (OXA)	*Enterobacteriaceae*, A. baumannii	Oxacillin, ampicillin cephalotin
	ESBL OXA-type		Penicillins, 3rd generation cephalosporins
	Carbapenemases (OXA)		Ampicillin, Imipenem, all β-lactams
B (Metallo-β-lactamases)	Metallo-β-lactamases (Carbapenemases) (VIM, IMP)	*Enterobacteriaceae* and nonfermenters	all β-lactams

* Characteristical resistance that are used for diagnostic purposes

The transfer of resistance genes from one bacterial cell to another can be explained by one of three mechanisms: conjugation, transformation or transduction (Coyle, 2015). Conjugation is mediated by plasmid DNA which replicates independently of the chromosomal DNA. When two bacteria is in a close environment together it is possible for the one bacterium to duplicates it plasmid DNA containing a resistant gene and transferring the fragment of DNA through the pilus, a bridge like structure, forming between the two cells. Transformation is when genes are transfer to other bacteria as “naked” DNA. When a nearby bacteria die and the DNA float in the environment, other bacteria can scavenge the loose or “naked” DNA and use genes to their advantage by incorporating it into

their own chromosomal DNA. Transduction is when bacterial DNA is transfered from one cell to another via a virus that infect bacteria. When a bacterial phage infect a bacterial cell, the phage interferes with the bacteria's DNA replication to produce more phages. Therefore, the bacterial DNA gets incorporated into the phage's DNA when the bacteria die, or cell lysis occurs. The phage then spreads and infects other bacteria (Coyle, 2015).

2.2.2.3.2. Other antibiotics use

Sulphonamides

Trimethoprim-sulfamethoxazole

The combination of Trimethoprim and Sulfonamides work on the metabolic pathway for folic synthesis in a bacteria cell. In many bacteria, para-aminobenzoic acid (PABA) is an essential metabolite in the synthesis of folic acid. Folic acid in turn is very important for the synthesis of nucleic acids. Sulfonamides are structural analogs similar to PABA that competes with PABA for the enzyme, dihydropteroate synthetase. In combination with Trimethoprim, which inhibits the enzyme, dihydropteroate synthetase, the folic acid synthesis pathway is blocked. This causes a bacteriostatic effect, blocking the bacteria to reproduce (Coyle, 2015).

Chloramphenicol

Chloramphenicol binds to the 50S ribosomal subunit that will cause interference with the binding of amino acids during protein synthesis. Therefore, the antimicrobial has an inhibitory effect in protein synthesis that causes cell death (Coyle, 2015).

Fluoroquinolones

Ciprofloxacin

This antibiotic is classified under the antibiotic class, Fluoroquinolones. Fluoroquinolones (e.g. Nalidixic acid, Ciprofloxacin, Levofloxacin and Gemifloxacin) interfere with the DNA synthesis of the cell by blocking the enzyme responsible for DNA synthesis, DNA gyrase. DNA gyrase help to unwind the DNA into single strands during DNA replication. When Fluoroquinolones binds the DNA gyrase, the single-stranded DNA is unable to wind back, and the broken DNA is released into the cell causing cell death (Coyle, 2015). Organisms have recently shown more resistance against fluoroquinolones in comparison to Carbapenems (Falagas & Karageorgopoulos, 2009). Fluoroquinolones were widely used in the clinical and veterinary drug because of its effectiveness to treat infections (Livermore *et al.*, 2002). The first resistance against Fluoroquinolones was observed in *Pseudomonas*, *E. coli* and *Enterobacteriaceae* in the 1990's. Amongst the Fluoroquinolones resistance, Ciprofloxacin resistance is relatively rare (Livermore *et al.*, 2002).

Tetracycline

Tetracycline is a broad-spectrum antibiotic that is effective against gram-negative and gram-positive bacteria (DeWaal *et al.*, 2012). In 2008, Kools *et al*. estimated that the antibiotics that are used the most in the veterinary medicine were Tetracycline, Beta-Lactam antibiotics and Sulphonamides. It is also the antibiotic used the most for growth promoters in the agricultural sector (Sarmah *et al.*, 2006). The global consumption of Tetracycline between the year 2000 – 2010 was about the 6th highest antibiotic consumed (Van Boeckel *et al.*, 2014). In the US and France, tetracycline makes up half of the total antibiotic usage for human and animals (Kools *et al.*, 2008). In Netherland, Tetracycline is the number one consumed antibiotic with a total use of 59% Tetracycline of the total antibiotics. The use of Tetracycline decreased from 2010 because of its well-known resistance (Van Boeckel *et al.*, 2014).

Tetracycline resistance was observed at poultry farms in the surface water, soil, broiler faeces and rinse water (Blaak *et al.*, 2015). Although tetracycline is used in the animal husbandry industry, resistance was detected in *Serratia fonticola* bacteria isolated from herbs in a study conducted by Nüesch-Inderbinen *et al.* (2015), suggesting that there is a link between animal husbandry and the cultivation of crops. This antibiotic is used in the agricultural sector to treat diseases with application in the poultry, cattle, sheep and swine sector (Chopra & Roberts, 2001). The use of Chlortetracycline in the swine industry is very common with a 40% usage rate in the US (Sarmah *et al.*, 2006). Antibiotics in the Tetracycline subclass is also used sub-therapeutically over a long period of time to improve the growth rate of animals. It is proven that a small amount of Chlortetracycline in the feed of chickens improve their growth rate and the effectiveness of feed administration (Chopra & Roberts, 2001). Tetracycline is used in aquaculture to minimize infections in salmon, catfish and lobsters. It is also used for the control of *Erwina amylovara*, a plant pathogen, on fruit trees and other plants (Chopra & Roberts, 2001). It is therefore clearly evident of how Tetracycline are introduced to the environment. It is traceable in surface water, soil and plants and it is therefore easy for bacteria to develop a resistance to the antibiotic (Heuer *et al.*, 2011).

Tetracyclines bind to the 30S of the ribosome that is responsible for the transfer of RNA. After that, new amino acids cannot attach to the growing protein chain and protein synthesis is blocked which is essential for cell function (Coyle, 2015). Because of this effect, the use of Tetracycline as a growth promoter in farm animals was banned in the early 1970's in Europe because of its contribution to the development of resistance in human isolates. Furthermore, when an bacteria contains the *tet(G)* gene displaying resistance against Tetracycline it is usually resistant to other antibiotics including Ampicillin, Chloramphenicol, Streptomycin, Spectinomycin and Sulphonamide (Chopra & Roberts, 2001). *Shigella,* commonly associated with fresh produce and irrigation water was first identified as a multidrug resistant organism in 1995 which was resistant to Tetracycline, Streptomycin and Chloramphenicol (Chopra & Roberts, 2001).

Aminoglycosides

Aminoglycosides are used as a broad-spectrum antibiotic because of its effectiveness on gram-positive and gram-negative bacteria with some limitations. Gentamycin is mostly used in the clinical sector whereas Neomycin is used as a veterinary antibiotic (Sarmah *et al.*, 2006). Aminoglycosides bind to the 30S ribosomal subunit and blocks synthesis in one of two ways or happens together: (i) the antimicrobial (in this case Gentamicin, Tobramycin, Amikacin or Streptomycin) bind to the 30S subunit and prevent the 30S unit to attach to the messenger RNA (mRNA) and/or (ii) the presence of the antimicrobial may cause that the mRNA is misread. This will cause the wrong amino acid to be inserted or will interfere with the correct amino acid to connect to each other that will with protein synthesis. The overall effect is that the cell will not function normally and will die off (Coyle, 2015).

2.3. Sources of contamination

2.3.1. Pre-harvest factors affecting the microbiological quality

There are many factors that play a role in the pre-harvest environment of fresh produce that contributes to the general microbiological load. However, there are several key factors that can contribute to the contamination of fresh produce that can directly influence the health of individuals that consume fresh produce. These factors are illustrated in a recent study conducted in Brazil. De Quadros Rodrigues *et al.* (2014) conducted interviews and questionnaires with the owners of lettuce producing farms to get insight into the implementation of good agricultural practices used. Additional to the questioners, De Quadros Rodrigues *et al.* (2014) also took samples from the field, soil, manure, irrigation water, washing water, workers' hands, equipment, lettuce seedlings and crops. According to this study, there was very little contamination on the seedlings of the crops. The irrigation water was contaminated with *E. coli* from 1.1 – 23 MPN.mL^{-1} and coliform counts from 12 – 23 MPN.mL^{-1}. Similar results were seen throughout the study. *E. coli* O157:H7 was only detected in irrigation water at one farm. The manure samples had high *E. coli* and coliform counts suggesting that the composting times were not appropriate. *Salmonella* was absent in the manure samples tested except for one manure sample at one farm. This indicates that the manure management was not sufficient for the control of foodborne pathogens in manure fertilisers (De Quadros Rodrigues *et al.,* 2014). Previous studies indicated that contaminated fertiliser may contaminate irrigation water and the soil that could have an impact on the microbiological safety of fresh produce (Johannessen, 2005). The questioners indicated that farms with trained technological and low staff turnover created less pressure for the implementation and maintenance of Good Agricultural Practices (GAP) because the employees know and understand their responsibilities.

Soil as a primary component in the production of fresh produce naturally has a great influence on the microbiological quality of fresh produce. Because fresh produce is consumed raw, it is a direct vehicle for contact with potential contamination soil. Various authors suggest that contaminated

irrigation water is the major source of pre-harvest contamination of fresh produce (Ailes *et al.*, 2008; Paulse *et al.*, 2012; Van Blommestein, 2012; Romanis, 2013; Lamprecht *et al.*, 2014). A 2012 study reported that there is a significant carry-over effect of pathogens from contaminated irrigation water to fresh produce (Van Blommestein, 2012). Many mechanisms of how the contamination irrigation water is affecting the fresh produce have been suggested over the years. Kroupitski *et al.* (2011) stated that the most *Salmonella* aggregates near the stomata of the lettuce. This study suggested that the most *Salmonella* contamination is caused by contaminated irrigation water that the plant utilises from the ground. Ge & Lee's (2015) results are similar. Kroupitski *et al.* (2011) stated that the internalised *Salmonella* is more concentrated in the lower parts of the plant such as the petiole. However, Honjoh *et al.* (2014) suggest two ways of contamination as a cause of irrigation water: Firstly, the weight of the irrigation water causes the plant leaves to touch contaminated soil. Secondly, the irrigation water can cause a splash effect that contaminates the fresh produce. Therefore, the above study implies that the outer leaves contain the highest concentration of microbial contamination. Taking into consideration all the above-mentioned mechanisms it valuable to analyse the crop (e.g. spinach or lettuce) as a whole to detect surface and internalised contamination. This approach will be beneficial because the consumer will be consuming all parts of the leafy green vegetable and will be affected by surface contamination as well as the internalised contamination. The pathogens present in water due to faecal contamination include *V. cholerae*, *S. typhi*, *Shigella dysenteriae*, *Campylobacter jejuni*, pathogenic strains of *E. coli* and the protozoan *Giardia lamblia* (Forsythe, 2010).

Bacteria can survive in soil for long periods of time depending on the soil composition, temperature, moisture (Honjoh *et al.*, 2014). The best conditions for bacteria survival are nutrient-rich soil and good moisture. *Salmonella* can survive up to 200 days in contaminated soil, even at low temperatures (Honjoh *et al.*, 2014).

Pesticides are used on crops to control plant diseases. It can be categorized into four main classes namely herbicides, fungicides, insecticides and bactericides. However, pesticides can also be a source of contamination (Dobhal *et al.*, 2014). According to Dobhal *et al.* (2014), there are a few ways how pesticides could add to the level of contamination of crops. Firstly, the pesticide itself can be contaminated with pathogens or the pesticide can be a stimulus for better growth (Ng *et al.*, 2005). The water used to dilute the pesticide can also be contaminated (Dobhal *et al.*, 2014). The time taken from the preparation of the pesticide and the application of the pesticide can also influence the growth of pathogens even if it was contaminated at a very low level (Dobhal *et al.*, 2014). The level of growth depends on the pesticide used and the pathogen it is contaminated with (Ng *et al.*, 2005).

2.3.2. Post-harvest factors affecting the microbiological quality

Post-harvest factors that affect the microbiological quality of fresh produce includes human handling, harvesting equipment, transport containers, wash & rinse water, sorting & cutting equipment, storage

and transportation (Olaimat & Holley, 2012). The seasonal effect also plays a role in the microbiological contamination level. Since the informal sector does not keep fresh produce at refrigeration temperatures, the warmer seasons creates a higher risk for microbiological contamination levels (Ailes *et al.,*2008)

De Quadros Rodrigues *et al.* (2014) reported the *E. coli* and coliform counts on the boxes used for harvesting and transport contained counts of less than 1.0 log cfu.cm $^{-2}$ for *E. coli* and 2.1 – 3.5 log.cm $^{-2}$ for coliforms that could contribute to the overall microbiological load on fresh produce. The containers used for the harvest and the transport of fresh produce are often not cleaned and sanitised properly which can give bacteria the opportunity to form biofilms (Srey *et al.*, 2013). The rinse water used for the washing of lettuce contained *E. coli* and coliforms after washing. If the washing water is not monitored regularly, this step could also add to the microbiological load or even be the processing step that introduces pathogens to the produce (De Quadros Rodrigues *et al.* 2014). The workers hands contained both *E. coli* and coliforms at levels of 1.0 – 1.9 log cfu.hand^{-1} for *E. coli* and 1.8 – 3.3 log cfu.hand^{-1} for coliforms (De Quadros Rodrigues *et al.* 2014). This indicates that the hands of the workers harvesting the crops can contribute to the overall microbiological load on the fresh produce.

2.4. Microbiological risk assessment

Policies, in general, have two objectives: (i) prevention of individuals consuming food that will cause harm to their health, (ii) promote the consumption of nutritious food to increase general quality of life (Josling & Roberts, 2018). These policies indirectly address the issues of food security and food safety. The enforcement of these policies is often challenging.

The South African microbiological guidelines are limited to the guidelines of the Department of Health (DoH, 2000). The only existing guidelines for fresh produce are stipulated as a non-compulsory regulation under the Guidelines for environmental health officers on the interpretation of microbiological analysis data of food (DoH, 2000). This regulation proposes, in Annexure B, a microbiological specification for fresh/cut vegetables that have a coliform count < 200 cfu.g^{-1}. The only pathogenic restrictions specified for fresh produce include *Salmonella* (0 cfu.g^{-1}) and *E. coli* (0 cfu.g^{-1}).

The European Food Safety Authority (EFSA), funded by the European Union works with, amongst others, the European Commission, Parliament and Council to establish and develop policy and regulatory procedures for the active management of food safety-related matters. On 24 January 2007, the scientific panel on Biological Hazards of the European Food Safety Authority stated their opinion that there is no direct correlation between *Salmonella* and *Enterobacteriaceae* (EFSA, 2007). In terms of fresh produce, this can be due to the natural amount of *Enterobacteriaceae* present on fresh produce that originated from the soil or water that was used for irrigation. Fresh produce is not a sterile product and contains naturally up to 1000 cfu.g^{-1} bacteria (Little *et al.*, 1999). The EFSA microbiological guideline for ready-to-eat fruit and vegetables propose a microbiological acceptable

range for *E. coli* of between 100-1000 cfu.g^{-1}. *E. coli* counts is the only proposed microbiological guideline for fruit and vegetables set out by the EFSA.

2.5. General conclusion

From literature, it is evident that there is a need for more evaluation studies into the microbiological safety of fresh produce especially in the informal markets in South Africa. Taking into consideration all the contamination opportunities the post-harvest environment can introduce (e.g. the handling of fresh produce, transportation vehicles, packaging, storage temperature etc.) as well as the level and frequency of contaminated irrigation water used in agriculture, the importance of monitoring the microbiological risk associated with fresh produce is obvious.

To only test for coliforms and *E. coli* is not sufficient because of the presence of other foodborne pathogens. The post-harvest environment creates an opportunity for contamination, time and ideal conditions for the survival and growth of foodborne pathogens. Awareness of antibiotic resistance in the environment is increasing and the spread of resistant organisms and genes are detected in fresh produce. Extended-Spectrum β-Lactamase producing *Enterobacteriaceae* is amongst the top priority antibiotic resistance list and its presence in fresh produce should also be evaluated. These results will give a better indication of the microbiological safety status of the fresh produce which is an important public health concern.

The aim of this study is to determine the microbiological safety risk of selected fresh produce sold at informal retailers at selected areas in Cape Town Metropolitan area, in South Africa, by testing for high-risk pathogens commonly associated with fresh produce. This will be achieved by enumerating indicator populations present on the selected fresh produce, and screening for high-risk pathogens including *Salmonella* spp., Shiga-toxin producing *E. coli* and *Listeria monocytogenes*. The selected fresh produce tested in this study includes lettuce, spinach, cabbage, tomatoes, green peppers and green beans. The presence and prevalence of Extended Spectrum β-Lactamase (ESBL) producing *Enterobacteriaceae* and their antibiotic resistant profiles, will also be evaluated. This will be achieved by initially screening for ESBL producers using ESBL ChromID (Biomerieux) agar and confirming ESBL status phenotypically with antibiotic disc diffusion test as well as genotypically. The antibiotic resistance profiles of the ESBL producers will be determined by exposing the organisms to eight antibiotics in different classes to confirm the possibility of multidrug resistance.

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CHAPTER 3

ENUMERATION OF HYGIENE INDICATOR MICROORGANISMS AND DETECTION OF SPECIFIC FOODBORNE PATHOGENS IN SELECTED FRESH PRODUCE SOLD AT INFORMAL RETAILERS IN THE CAPE TOWN METROPOLITAN AREA

3.1. SUMMARY

The purpose of this study was to determine the microbiological safety of fresh produce sold at the informal market in the Cape Town Metropolitan area, South Africa, by enumerating hygiene indicator systems such as Coliforms, *Escherichia coli* and *Enterobacteriaceae*. Five informal vendors were selected to represent the informal market. Two different products were selected for sampling (five repetitions of each product). Lettuce was always selected as one product and the other product varied between cabbage, spinach, green peppers, tomatoes or green beans. Each site was visited three times. Indicator systems alone, however, do not give an indication of the presence of specific pathogens. Thus, the three most prevalent bacteria causing foodborne illnesses associated with fresh produce were also tested including *Salmonella* spp, *Listeria monocytogenes* and Shiga toxin producing *E. coli* (STEC). The hygiene indicator counts for all the produce at all the sites where well over the advised microbiological limits according to the Department of Health of South Africa. No *Salmonella* or *Listeria monocytogenes* were detected in any of the fresh produce, however, one lettuce sample tested positive for STEC. Out of a total of 150 produce samples tested, 11.33% contained *E. coli* at average levels of 2.7×10^3 cfu.g^{-1}. There were no significant differences ($p < 0.05$) between the presence of *E. coli* in the different fresh produce samples tested. The presence of *E. coli* occurred sporadically suggesting that *E. coli* contamination could be linked to the post-harvest handling of fresh produce. Regardless of the high hygiene indicator counts and the sporadic presence of *E. coli,* no pathogens were detected (excluding one event). Therefore, there is no evidence supporting the assumption that the fresh produce tested is unsafe for consumption.

3.2. INTRODUCTION

The evaluation of the microbiological safety of fresh produce is essential in the process of ensuring safe food. The number of foodborne outbreaks related to fresh produce has seen an increase over the past four decades as reported by the Centre of Disease Control and Prevention's (CDC) Foodborne Disease Outbreak Surveillance System that started in 1973 (Herman *et al.*, 2015). Since then, 606 outbreaks were associated with leafy green vegetables up to the year 2012. The pathogens that accounted for 55% of the outbreaks was the norovirus, 18% was attributed to Shiga toxin-producing *E. coli* (STEC) and 11% *Salmonella*. Out of the 20 003 reported cases, 1 030 were hospitalised and 19 deaths were reported. The World Health Organisation (WHO), CDC and the Food and Agricultural Organisation of the United Nations (FAO) are all in agreement that leafy green vegetables are the food type that causes the most foodborne disease outbreaks. As a result, it classified as the level one priority food type. This decision is based on the frequency and severity of microbiological contamination, size of the production of the crop, the complexity of the production chain, the potential for the amplification of foodborne pathogens in the food chain and the extent of international trade and the economic impact (FAO & WHO, 2008). Leafy greens includes spinach, cabbage, lettuce and salad leaves, and fresh herbs. Tomatoes, although not a leafy green vegetable, are also largely consumed raw and are classified as level two priority. It is one of the top 10 most consumed vegetables in SA (STATSSA, 2002).

Fresh produce can be contaminated as a result of a variety of pre- or post-harvest factors. Pre-harvest factors that influence the microbiological quality of the fresh produce include animal activity on the farm or adjacent land, climatic conditions, seasons, manure (wild or domestic animals) and soil, growing and harvesting equipment, insects, pests and irrigation water (Gil *et al.*, 2015). Irrigation water is one of the major contributing factors to contaminated fresh produce (Pachepsky *et al.*, 2011). The majority of South African irrigation water is surface water either from rivers or dams which increases the risk for pathogens in the water (Ashton, 2007). Runoff from contaminated soil, agricultural land used for livestock, informal settlements, industrial factories and sewage are a few factors contributing to pathogens in surface water (Beuchat, 2002; Gil *et al.*, 2015). Post-harvest factors influencing the quality and safety of the fresh produce include handling, sorting, size reduction and cutting, potentially washing, packaging, transporting and storage (Gil *et al.*, 2015).

To ensure food safety, there is regulations and policies in place to manage the food safety risk at farms (pre-harvest) and after harvest when the produce is packed, transported and stored before it is distributed to retail outlets for the consumer. GLOBAL GAP is an international farm management standard that ensures the requirements of Good Agricultural Practices (GAP) before harvest. ISO 22 000:2005 and ISO 9001 regulates food safety risk after harvest. SANS 10049:2012, a revised standard for the management of food safety for the requirements for prerequisite programs stated that food handlers are expected to meet the minimum requirements as required by consumer and regulatory authorities. Food handlers in food handling areas, according to the SANS

10049:2012, include farms, pack houses, fresh produce markets, manufacturing facilities, factory shops, catering units and kitchens, restaurants, butcheries, retailers, distribution centres and transporting vehicles. However, informal street vendor trading does not adhere to this standard – it is not practical in the informal sector. The international alliance for street vending organisation has warned that street vendors has no best practises policies to control the food safety risk (Skinner, 2008). The fresh produce is typically handled, transported and stored at the informal trader's best knowledge with no formal handwashing facilities or water infrastructure available. Microbiological contamination is, therefore, expected.

To evaluate the microbiological safety of fresh produce it is necessary to determine the overall microbiological quality and also to test for specific foodborne pathogens. Indicator organisms are used to determine the overall hygiene of a sample by testing for *E. coli* and coliforms which are two indicator organisms most frequently used (Forsythe, 2010). *E. coli* is an indicator of faecal contamination either from animal manure or human faecal sources. Coliforms are a good indicator system used to determine overall hygiene. A shortcoming of coliforms, however, is that it only accounts for a smaller gram-negative organism group and does not include some major foodborne pathogens of concern. Therefore, another indicator system is used, *Enterobacteriaceae*. It is an indicator of food safety rather than just overall hygiene (Forsythe, 2010). Unfortunately, there is no recommended microbiological guideline in South Africa for *Enterobacteriaceae* and therefore coliforms are still most frequently used. *Enterobacteriaceae* alone is not sufficient to determine food safety of fresh produce, therefore, it is necessary to test for high risk foodborne pathogens as well in addition to the overall hygiene (Busta *et al.*, 2003).

The aim of this study was to enumerate hygiene indicator microorganisms such as coliforms, *Enterobacteriaceae* and *Escherichia coli* in selected fresh produce sold at informal vendors in the Cape Town Metropolitan area. The safety of fresh produce was determined by the detection of specific foodborne pathogens including *Salmonella* spp., STEC and *Listeria monocytogenes*.

3.3. MATERIALS AND METHODS

3.3.1. Validation of the pathogen detection method

In this study, seven different microbiological tests were performed on the same 25 g sample including the enumeration of coliforms, *Enterobacteriaceae* and *E. coli* plus the detection of *Salmonella*, STEC and *L. monocytogenes*. The isolation of Extended Spectrum β-Lactamase (ESBL) producing *Enterobacteriaceae* is discussed in Chapter 4. For the preparation of test samples with the purpose of enumeration and detection, a 25 g sample is weighed and macerated in a 225 mL diluent (SANS, 2004). For the enumeration of *E. coli,* coliforms and *Enterobacteriaceae* Buffered Peptone Water (BPW) or Ringer solution can be used to macerate and prepare a dilution series (SANS, 2004). For the detection of ESBL producing *Enterobacteriaceae*, however, BPW should be used in the initial

maceration step and incubated for 24 hours at 37˚C (Zurfluh *et al.*, 2015). However, for the detection of *Salmonella*, STEC and *L. monocytogenes* the BAX® system is used with customised kits. According to the manufacturer's instructions the BAX® system MP® enrichment media is recommended for the use in the pre-enrichment step for the detection of *Salmonella, L. monocytogenes* and STEC. In this study, a single enrichment medium had to be chosen that was both suitable for the enrichment step of all pathogen detection tests using the BAX® system and also for the enumeration tests that did not require an enrichment step. For this purpose, BPW was selected as the primary enrichment medium for the detection of the pathogens instead of the BAX® system MP media (recommended by the BAX® system manufacturer). Therefore, to standardise the primary maceration/enrichment step for all microbiological tests done in this study, a validation procedure was performed to determine whether BPW is compatible with the BAX® system kits.

Pathogen detection system

The BAX® Q7 system (Hygiena) was used for the detection of *Salmonella* spp., STEC and *Listeria monocytogenes.* This PCR based system is a rapid molecular pathogen detection system that handles both real-time and end-point assays. The BAX® system results are available 24 hours after sample incubation and makes use of internal controls with every assay to validate negative results. Positive control cultures were selected for the validation process and included *Salmonella typhimurium* ATCC 14028 as well as an *E. coli* (STEC) strain previously isolated from meat and *Listeria monocytogenes* isolated from a food processing environment (P. Gouws, 2017, Professor, Department of Food Science, Stellenbosch, South Africa, personal communication, 22 May.)

Method for preparing the positive control

Each positive control, previously stored in 40% glycerol ($v.v^{-1}$) at - 80˚C, was defrosted and ± 10 µL was transferred to 10 mL sterile Tryptone Soy Broth (OXOID) and incubated for 24 hours at 37˚C. The bacterial culture was then concentrated by transferring 1 mL to a 1.5 mL tube and centrifuged (Neofuge 13, Vacutec) for 1 min at 12 000 rpm. The supernatant was discarded and 900 µL Ringer solution (Merck) was added to the pellet and vortexed before repeating centrifugation. The wash process was then repeated twice after which the pellet was suspended in 1 mL Ringer solution. In total, the culture was washed three times and resuspended after the third washing process.

Determining the initial concentration of the prepared positive control

The positive controls were all spectrophotometrically standardised to a pre-determined optical value (Table 3.1). The absorbance of each positive control was first measured at 600 nm and recorded as the optical density (OD) of the cells using a Merck Spectroquant Prove 600 spectrophotometer. After the OD value (C_1) of the positive controls was determined, the equation $C_1V_1 = C_2V_2$ was used to standardise the cells at an OD value in Table 3.1.

C_1 = OD concentration (Determine with spectrophotometer reading at 600nm

C_2 = Desired OD concentration e.g. 0.1 OD

V_2 = End volume e.g. 1.5 mL

$V_1 = \frac{C_2 V_2}{C_1} = x$ mL

The required volume (x mL) was suspended in (1.5 mL – x mL) Ringer solution to make up the final OD concentration specified in Table 3.1.

Table 3.1 The optical density correlating to an estimated cfu.g^{-1} count of the positive controls

Isolate	Optical density (OD)	Corresponding cfu/mL	Reference
L. monocytogenes	0.1	1x10^5	(Wang *et al.*, 2015)
Salmonella	0.2	1x10^8	(Gorski, 2012)
E. coli (STEC)	0.7	7x10^8	(Nilsson *et al.*, 1997)

Enumeration of the standardised positive controls

The standardised positive control suspensions were enumerated using nutrient agar (Biolab) and standard plate count methods. Plates were incubated for 24 hours at 37˚C. The colonies were counted and reported as cfu.mL^{-1}.

Spiking with positive controls

Butter lettuce (cut & pre-washed) was purchased from a trusted and good quality supplier to minimise the amount of background microflora on the test sample. Two portions (2 x 25 g) were kept for a negative control and were not spiked. The positive control cultures were freshly prepared as described previously. To determine the BAX® system's limit of detection when BPW is used as pre-enrichment step, lettuce samples were spiked with cell concentrations in the log range of 10^0-10^1, 10^1-10^2 and 10^2-10^3. Six previously weighed lettuce samples (25 g) were spiked with a small volume (to allow quicker air drying) of the culture that corresponds to the desired spiking dose needed (Figure 3.1). This was done in duplicate. In total, eight samples were prepared for each pathogen validation trial: one (negative) sample that was not spiked (duplicate), one sample containing 10^0-10^1 spiking dose (in duplicate), one sample containing 10^1-10^2 spiking dose (in duplicate) and one sample containing 10^2-10^3 spiking dose (in duplicate).

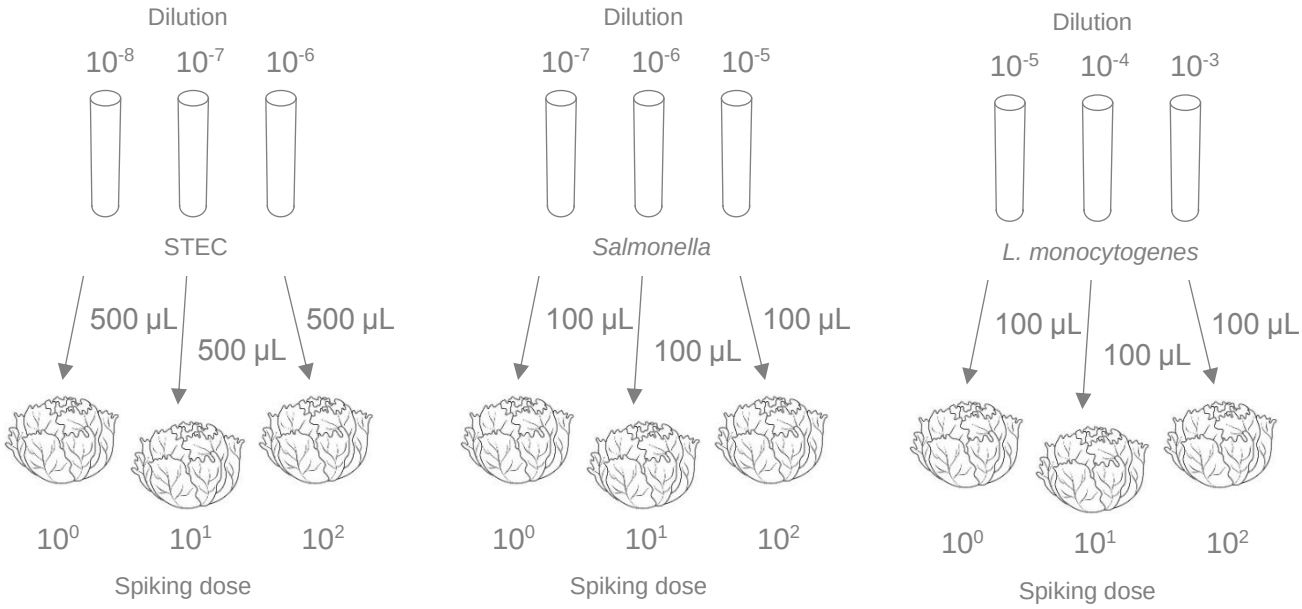

Figure 3.1 A visual illustration of the quantities used from the respective standardised positive control cultures to achieve for the desired spiking dose in the range 10^0-10^1, 10^1-10^2 and 10^2-10^3.

Validation: Detection of positive controls

The 25 g spiked sample was homogenised in BPW (225 mL) in a stomacher bag mixer® (Interscience) for 2 min. The samples were incubated for 24 hours at 37˚C after which the samples were prepared and tested according to the BAX® system (Hygiena) manufacturer's instructions.

3.3.2. Pathogen detection and isolation

The detection of *Listeria monocytogenes, Salmonella* spp. and STEC was done using the BAX® System (Hygiena) and the appropriate BAX® assay kits (Microsep) according to the manufacturer's protocol. The appropriate BAX® system kit used for the detection of the pathogens is listed in Table 3.2. If the detection result was positive, the isolation process for each pathogen was followed.

Table 3.2 The BAX® system compatible kits used for the detection of the appropriate pathogens

Pathogen being tested	**Appropriate BAX® system kit**
Shiga toxin producing *E. coli*	Real Time *E. coli* STEC (screening *stx* & *eae*) kit
Salmonella spp.	*Salmonella* 2 assay kit
Listeria monocytogenes	*L. monocytogenes* 24E assay kit

L. monocytogenes isolation

The RAPID'L.mono method used for the isolation of *Listeria monocytogenes* and other species has been certified by NF validation as an alternative method to the standard ISO 11290-1 (*Detection of Listeria monocytogens and other species of Listeria spp in all food products for human consumption and in environmental samples*) according to the ISO 16140 protocol for method validation. The RAPID'L.mono method is used according to the protocol of the manufactures (Bio-Rad, 2014). A pre-weighed 25 g sample was macerated in 225 mL Frazer broth (OXOID) enriched with half Frazer supplement (OXOID) and incubated for 24 hours at 30˚C. The sample was streaked onto RAPID'L.mono agar (Bio-Rad) in duplicate and incubated for 24 hours at 35˚C. Any colonies displaying a black centre were considered as presumptive *Listeria monocytogenes.*

Salmonella spp. isolation

The isolation of *Salmonella* spp was done by following the protocol laid out in the Bacteriological Analytical Manual governed by the Food and Drug Administration (Andrews *et al.,* 2009). The pre-weighed 25 g sample was macerated in 225 mL BPW and incubated for 24 hours at 35˚C. After the incubation, 0.1 mL was transferred to 10 mL RV broth (OXOID) and incubated for a further 24 hours at 42˚C. This was then streaked out in duplicate onto XLD agar (Merck) and Hektoen agar (OXOID) and incubated for 24 hours at 35˚C. The small black colonies on XLD agar and dark green colonies on Hektoen agar are presumptive *Salmonella.*

E. coli (STEC) isolation

The method followed for the isolation of *E. coli* is described in Feng & Weagant (2009). The pre-weight 25 g sample was macerated in 225 mL BPW and incubated for 24 hours at 35˚C. After the incubation, 1 mL of the sample was transferred to 9 mL EC broth (OXOID) and incubated for a further 24 hours at 35˚C. The incubated EC broth sample was streaked out in duplicate onto L-EMB agar (OXOID) and incubated for 24 hours at 35˚C. The isolated colonies were tested again to confirm using the BAX® system.

3.3.3. Experimental study

3.3.3.1. Site selection

In this study the microbiological safety of selected fresh produce was determined at informal retailers. Informal retailers, by own definition, are street vendors that sell fruit and vegetables in a self-constructed (but permanent) structure in an uncontrolled environment. Private transport is used and the source of the fresh produce is from various fluctuating sources without any formal transactions which could later be used for traceability purposes. Figure 3.2 is an example of a typical street vendor. In South Africa, there are a large number of informal vendors, which are estimated to be about 10% of the overall food market trade (Skinner, 2008). The site selection was done in with Cape

Town Scientific Services (CTSS) which was essential for representative informal street vendor data collection in the Cape Town Metropolitan area. Five sites (Table 3.3) were selected by the CTSS based on information collected from the street vendors via surveys previously conducted by CTSS. According to the CTSS the fresh produce is sourced from various nearby farms or the Epping fruit and vegetable market (S.D. Ariefdien, 2017, Senior Environmental Health Practitioner, Klipfontein sub-District, South Africa, personal communication, 3 November).

Figure 3.2 The inside of an informal vendor stand.

There were no formal food safety complaints linked to any of the five sites. All five sites have permits from the Economic Development Department and have been trading for decades (S.D. Ariefdien, 2017, Senior Environmental Health Practitioner, Klipfontein sub-District, South Africa, personal communication, 3 November).

Table 3.3 Location of the selected five informal vendor sites in the Cape Town metropolitan area

Site	Area
A	Delft
B	Mitchells Plain
C	Gatesville
D	Rylands
E	Epping

3.3.3.2. Sample frequency

Two produce types (five samples each) were collected at each site. The one product was always lettuce and the other product was selected based on the availability at the site at that time. The other fresh produce products sampled included any of the following: cabbage, spinach, green beans, green peppers or tomatoes. In total ten samples were collected at each site. One site was sampled each week. After all five sites (Site A-E) were sampled once over a time period of five weeks, the same pattern was repeated to have a total of three repetitions for each site. Three-week time lapses were left between repetitions which resulted that a site was visited every eight weeks.

3.3.3.3. Sample collection and preparation

At each site, the samples were collected and placed in plastic bags. All samples were placed in a cool box and transported to the laboratory to be stored at 4˚C until analysed within 24 hours. Upon arrival, the fresh produce samples were placed in individual plastic bags and given a unique randomised number. Each sample was sliced on its own pre-sterilised metal cutting board with a sterilised knife. Figure 3.3 shows how different pieces of the respective fresh produce were selected to make up a 25 g represenative sample. All samples were weighed out (25 g) in stomacher bags and stored at 4˚C before analysis took place the day after the samples were collected. Before analysis, the 25 g sample was macerated in 225 mL sterilised buffered peptone water (Merck) for 2 minutes at 230 rpm in a 220V Interscience Bag Mixer.

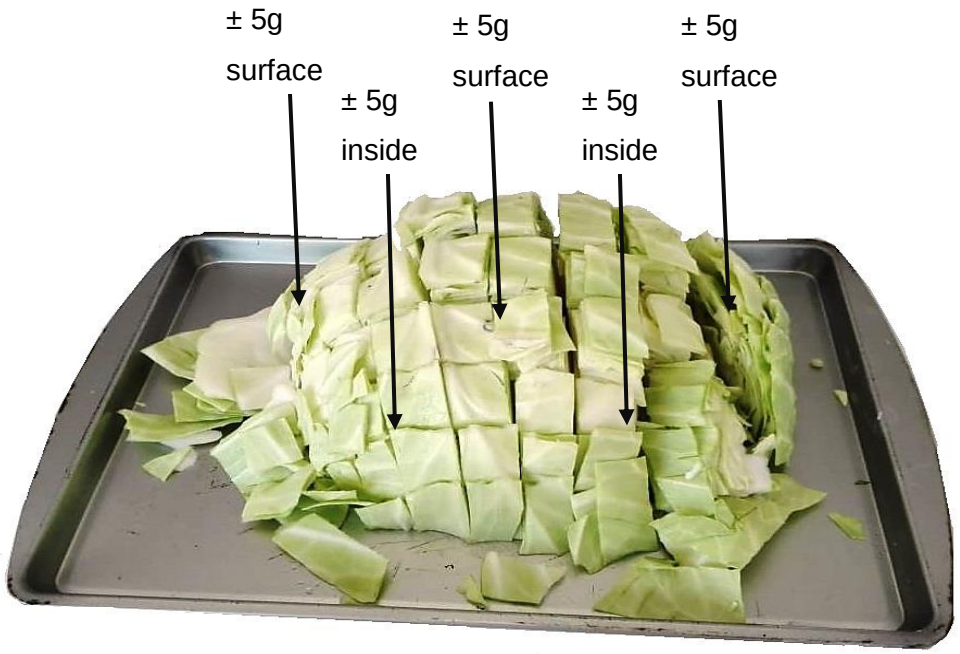

Figure 3.3 An example of how the fresh produce is sliced on a sterile cutting board. Twenty-five grams of sliced leaves was selected systematically before maceration.

3.3.3.4. Sample analysis

Hygiene indicators

E. coli is a good indicator organism for faecal contamination and indicates the possible presence of enteric pathogens (Forsythe, 2010). Other hygiene indicator systems were also used to evaluate the overall microbiological safety of fresh produce tested in this study. These included coliforms and *Enterobacteriaceae* that are two groups of indicator organisms frequently used. (Forsythe, 2010).

Enumeration of Coliforms, E. coli and Enterobacteriaceae

The RAPID'*E. coli* 2 method that was used for the enumeration of coliforms and *E. coli* has AFNOR approval as a valid alternative to the NF ISO 4832 (standard for the enumeration of coliforms) and NF ISO 16649-2 (enumeration of β-Glucuronidase-positive *E. coli*) according to the ISO 16140 protocol for method validation. The RAPID'*E. coli* 2 method was used according to the manufacture's (Bio-Rad, South Africa) protocol. The 25 g sample was macerated with 225 mL of BPW and a dilution series (10^{-2} - 10^{-6}) was prepared. The dilution series were plated out in duplicate (pour plate technique) using RAPID'*E. coli* 2 agar (Bio-Rad, South Africa) and incubated for 24 hours at 37˚C. After incubation, the plates that had a count between 30 - 300 were counted and reported. The chromogenic RAPID'*E. coli* 2 agar (Bio-Rad, South Africa), distinguish between coliforms and *E. coli* based on different colour reactions. The colonies displaying a blue colour were considered presumptive coliforms and the colonies displaying a pink-violet colour, presumptive *E. coli* (Bio-Rad, 2014).

The enumeration of *Enterobacteriaceae* was based on SANS 21528-2:2005. The same preparation process was followed as for the enumeration of *E. coli* and coliforms. The macerated fresh produce samples were plated out (pour plate technique) in duplicate on VRBG (Merck) agar followed by 24-hour incubation at 37˚C. The plates that have colony numbers between 30-300 was enumerated and reported (SANS, 2008).

Pathogen detection and isolation

The detection of the respective pathogens was done using the BAX® system (Hygiena) and its respective assay kits. Isolation of pathogens was done using standard isolation methods (described in the validation of the pathogen detection methods section).

Statistical analysis

The statistical analysis including both the calculation of the means, standard deviations and the construction of bar graphs were completed using Sigma Plot version 13 software. The variance estimation and precision Analysis Calculation (VEPAC) to determine the least significant differences

was done by using Statistica 13.0 software. A 95% confidence interval is used to determine significant differences ($p < 0.05$).

3.4 RESULTS AND DISCUSSION

3.4.1 Validation study

A single diluent (BPW) was selected for both pathogen detection and the enumeration of hygiene indication organisms. Since BPW was used in this study with the BAX® system instead of the recommended MP® media, a validation of the pathogen detection method (application of the BAX® system and kits) was necessary.

The standardised positive controls were enumerated on Nutrient agar (Biolab) since the positive controls are pure cultures and easily grow on nutrient agar. The counts (Table 3.4) indicate that the spiking dose of the positive controls were accurately determined, since the counts fall in the desired spiking dose range. The positive control, *L. monocytogenes*, was an exception and resulted in a higher spiking range than desired. This, however, had a positive effect on the validation study because the detection limit for *L. monocytogenes* turned out to be very high and the study did not make provision for detection limits higher than 1000 cfu.g^{-1}.

Table 3.4 The chosen spiking dose log ranges and the actual count that the lettuce samples were spiked with

	Spiking dose log ranges (cfu.mL^{-1})		
Positive control	10^0-10^1	10^1-10^2	10^2-10^3
E. coli (STEC)	2	31	260
Salmonella	1	15	110
L. monocytogenes	45	210	TNTC

The results of the spiked samples are presented in Table 3.5. The BAX® system is a detection system that gives a presence or absence result, the system cannot enumerate. The purpose of this validation was to test the sensitivity of the BAX® system with the new diluent (BPW). All the spiked positive

Table 3.5 The results generated by the BAX® system indicating a presence (+) or absence (-) of the specific positive control (spiked sample) at the known spiking dose

	Spiking dose log ranges (cfu.mL^{-1})			
Positive control	10^0-10^1	10^1-10^2	10^2-10^3	Negative control
E. coli (STEC)	+	+	+	-
Salmonella	-	+	+	-
L. monocytogenes	-	-	+	-

controls should generate a positive result. However, when the BAX® system gives a negative result, it indicates that the system is not sensitive enough to detect pathogens below certain levels. Depending on the sensitivity of the system towards a specific pathogen the result can be refined to: <10, <100 or <1000.

The STEC results (Table 3.5) indicated that the sensitivity of the BAX® system towards STEC detection is very high. The lowest detection limit for this specific environmental strain of STEC was between 10^0-10^1 cfu.mL^{-1} or more specifically > 2 cfu.mL^{-1}. After the STEC samples were detected using the BAX® system, the isolation process was completed. After the inoculated STEC samples were isolated, the isolated colonies were subjected to the BAX system again to confirm that the organisms isolated were STEC.

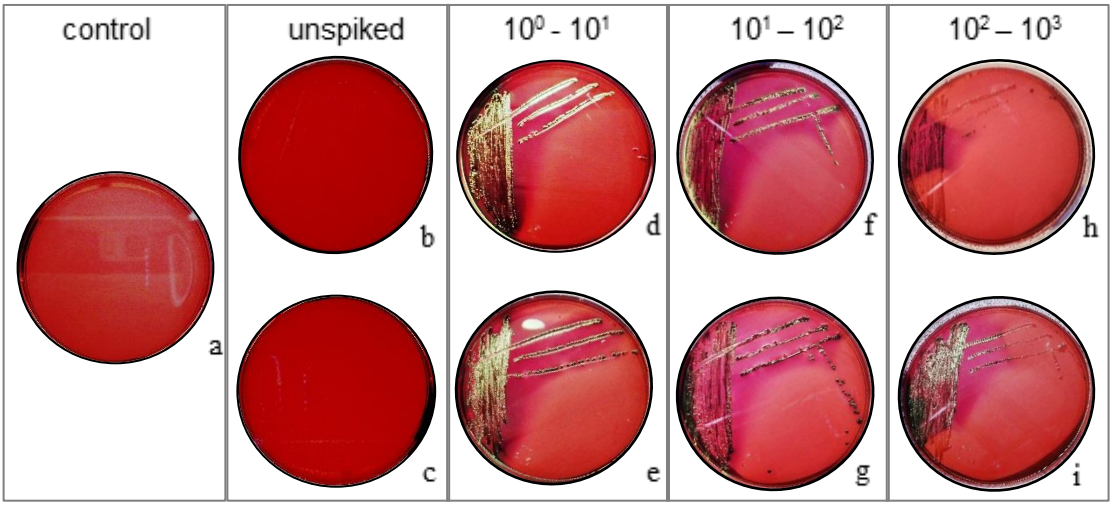

Figure 3.4 The images of the L-EMB agar plates used for the isolation of *E. coli* (STEC) in duplicate: (a) clear agar negative control, (b&c) unspiked sample control, (d&e) STEC 10^0-10^1, (f&g) STEC 10^1-10^2, (h&i) STEC 10^2-10^3.

Figure 3.4 shows the results of the isolation process of STEC. The LEMB agar negative control plate (a) and the unspiked control plates (b &c) were clear. The 10^0-10^1, 10^1-10^2 and 10^2-10^3 spiking dose range samples and mixed positive control culture could be isolated successfully. L-EMB agar is a selective agar for *E. coli* that grows as a green metallic sheen colony.

The sensitivity of the BAX® system towards the specific strain of *Salmonella* was less sensitive in comparison to the STEC detection sensitivity. The minimum detection limit for *Salmonella* was between 10-100 cfu.mL^{-1}. Or more specifically > 15 cfu.mL^{-1} according to the results in Table 3.4. Figure 3.5 shows the isolation of *Salmonella* on XLD and Hektoen agar. The isolation and the BAX® system results supported the same conclution. The dose 10^0-10^1 could not be successfully isolated using XLD or Hektoen agar nor detected by the BAX® system. Overall the sensitivity of Hektoen agar was more favourable. *Salmonella* could be detected with the BAX® system and be isolated from the mixture of pathogens in the spiking dose of > 15 cfu. The *Salmonella*

are represented by the black colonies on the XLD agar. The black colonies (*Salmonella)* often only appeared at the very end tip of the streak. The colonies with the salmon orange colour in the Hektoen agar are possibly *Shigella* whereas *Salmonella* is a dark green colony. It was easier to detect *Salmonella* on Hektoen agar because the colonies of interest were better visually shown.

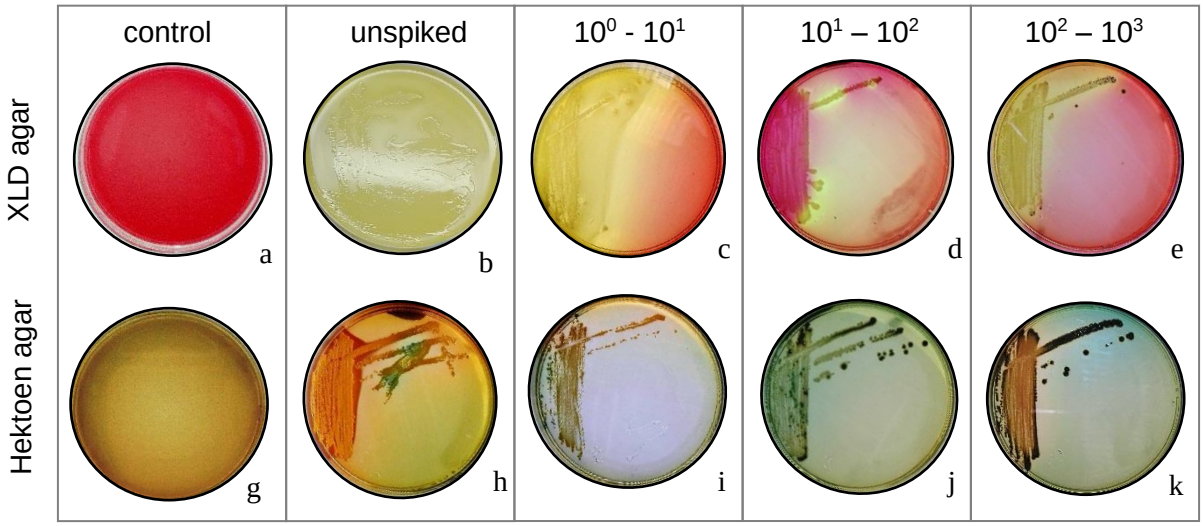

Figure 3.5 The images of the isolation plates used for the isolation of streaked onto XLD agar (a-e) and on Hektoen agar (g-k): (a&g) clear agar negative control, (b&h) Sample negative control, (c&i) *Salmonella* 10^0-10^1, (d&j) *Salmonella* 10^1-10^2, (e&k) *Salmonella* 10^2-10^3.

Therefore, the detection limits of *L. monocytogenes* are between 10^3 – 10^4, *Salmonella* 10^1 – 10^2 and STEC 10^0 – 10^1 which are the detection limits of the respective foodborne pathogens in this study. The validation determined the sensitivity of the BAX® system when the enrichment diluent is changed to BPW. The validation study is completed with one strain of each positive control and therefore limiting the results to the specific strain selected. Thus, the results of the validation study will only be suitable for this research project.

3.4.2 Experimental study results and discussion

This study focused on the microbiological safety of fresh produce specifically in the informal sector. This makes it challenging to conclude, with certainty, what the cause of the contaminated products were because the informal trader's source fresh produce from various sources. The fresh produce was also not sourced from the same source. Therefore, even survey information regarding the source of fresh produce sold at a vendor is only a vague representation. Further microbiological contamination depends on the specific farm where the fresh produce was produced and how it was produced, how it was transported, in which conditions it was displayed before it was bought by consumers, how it was handled at the informal vendor and lastly how the consumer handled the

unpackaged fresh produce before consumption. Due to limited information, it is challenging to clarify the high or low coliform/*Enterobacteriaceae* and *E. coli* counts.

3.4.2.1 Sample distribution

The number of samples collected at each site was kept constant, however, the second product varied according to the availability of fresh produce types. Figure 3.6, Figure 3.7 and Figure 3.8 showed the sample distribution over the five sites (site A - E) of all three repetitions respectively. The amount of each product is expressed as a percentage of the total samples collected for each repetition.

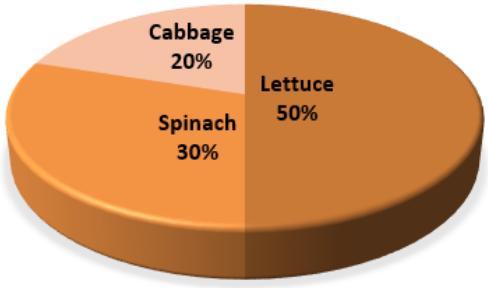

Figure 3.6 The sample distribution of repetition 1 over sites A – E. The total amount of samples in repetition 1 is 50 samples.

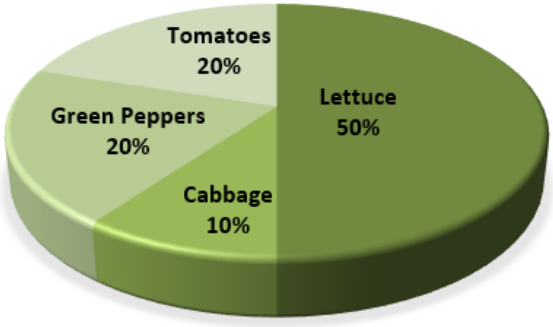

Figure 3.7 The sample distribution of repetition 2 over sites A – E. The total amount of samples in repetition 2 is 50 samples.

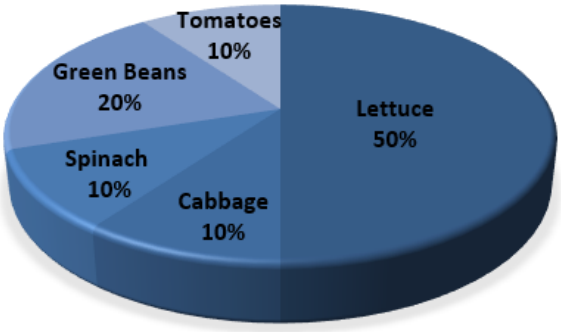

Figure 3.8 The sample distribution of repetition 3 over sites A – E. The total amount of samples in repetition 3 is 50 samples.

3.4.2.2 Food safety at the selected informal vendors

To determine the general microbiological safety of fresh produce at sites A – E, coliforms were used as hygiene indicators. All five repetitions of a product were enumerated including lettuce and the other fresh produce type of choice for all five sites. At site A and B, lettuce and cabbage was selected. Lettuce and spinach were selected at sites C, D and E. The bars below the zero x-axes give an indication of the products that were not tested. The microbiological limit advised from the DoH is also indicated.

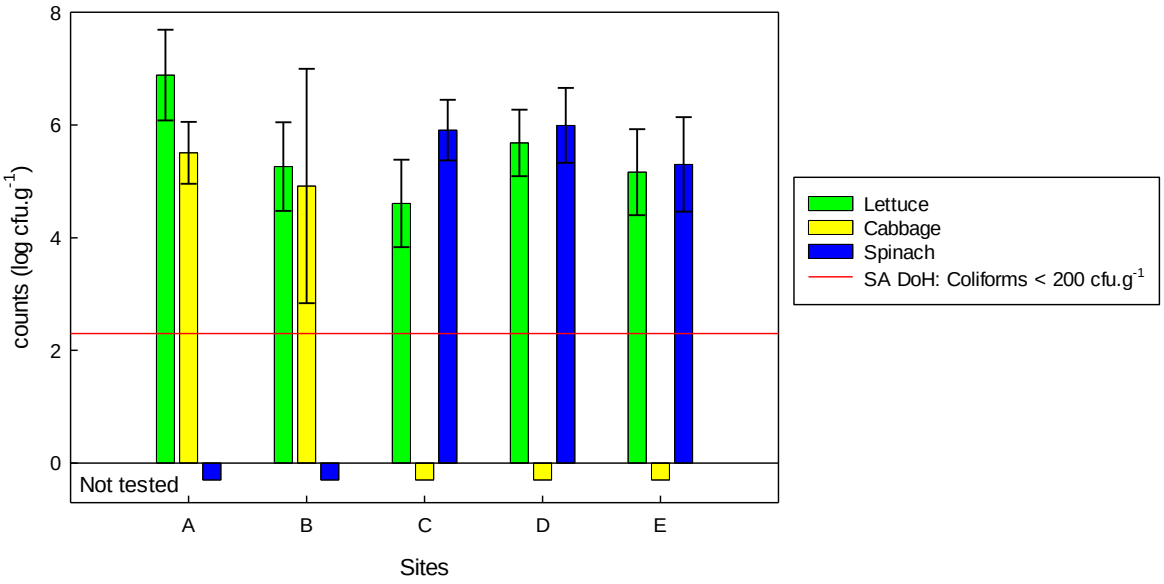

Figure 3.9 The total coliform counts of the selected fresh produce products sampled at sites A – E for repetition 1 of 3.

The coliform counts for lettuce, spinach and green beans were well over the advised DoH microbiological limit of < 200 cfu.g^{-1} (DoH, 2000) (Figures 3.9 – 3.11). The tomatoes and green pepper's coliform counts in figure 3.10 showed variation from the rest of the products during repetition 2. At site A the coliform count for green peppers was above the DoH microbiological limit, however, at site B the counts were within the DoH's limits. Because very little is known about the origin or handling of the product, the reasons for the results can only be speculated about. Similar results were observed for the tomatoes (Figure 3.10). Site D's coliform results were within the DoH's microbiological limit whereas site E's coliform results for tomatoes was above the microbiological limit. The average range for coliforms on cabbage over all three repetitions (Figure 3.9 – 3.11) was 4.24 – 6.89 log cfu.g^{-1}. A similar study completed in 2017,sampled fresh produce from the formal retailers and informal vendors in Johannesburg, South Africa (Du Plessis *et al.*, 2017). This formal study documented a range of 2.78 – 5.73 log cfu.g^{-1} coliforms for cabbage sampled at six different informal retailers (Du Plessis *et al.*, 2017). In this study however, the average coliform count for cabbage was slightly higher.

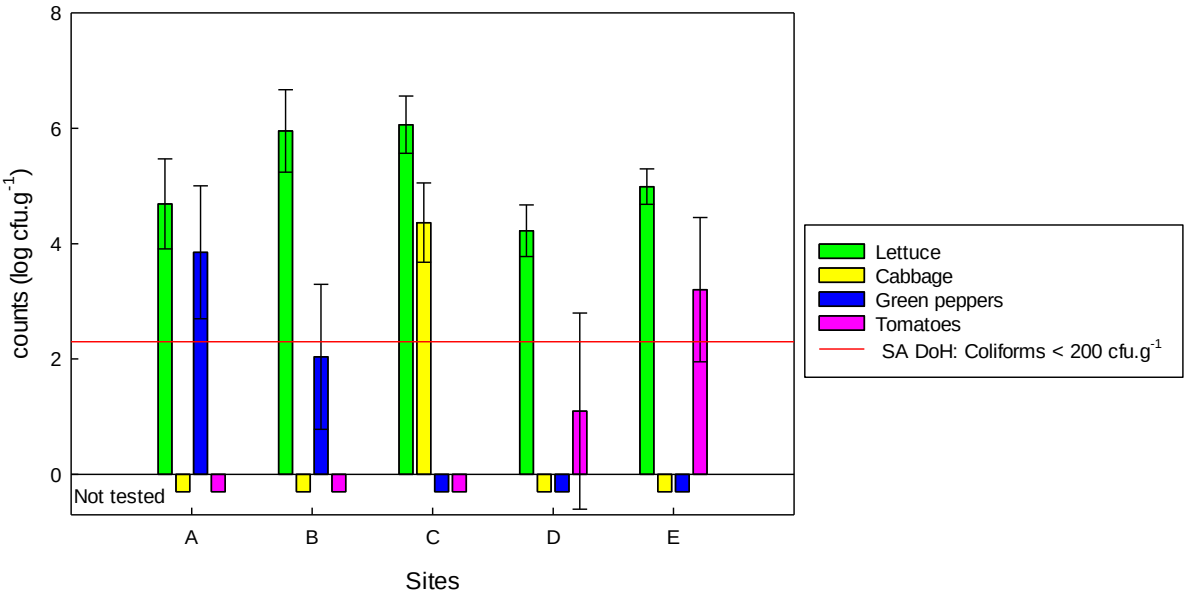

Figure 3.10 The total coliform counts of the selected fresh produce products sampled at sites A – E for repetition 2 of 3.

The average coliform count range for spinach over all three repetitions was 5.13 – 5.99 log cfu.g^{-1} (Figure 3.9 - 3.11) whereas the results from a similar study conducted in Johannesburg reported an average coliform count range of 2.64 – 5.74 log cfu.g^{-1} from spinach samples sold at informal retailers (Du Plessis *et al.*, 2017). The spinach results from both studies are very similar except that this study's spinach coliform counts were more consistent.

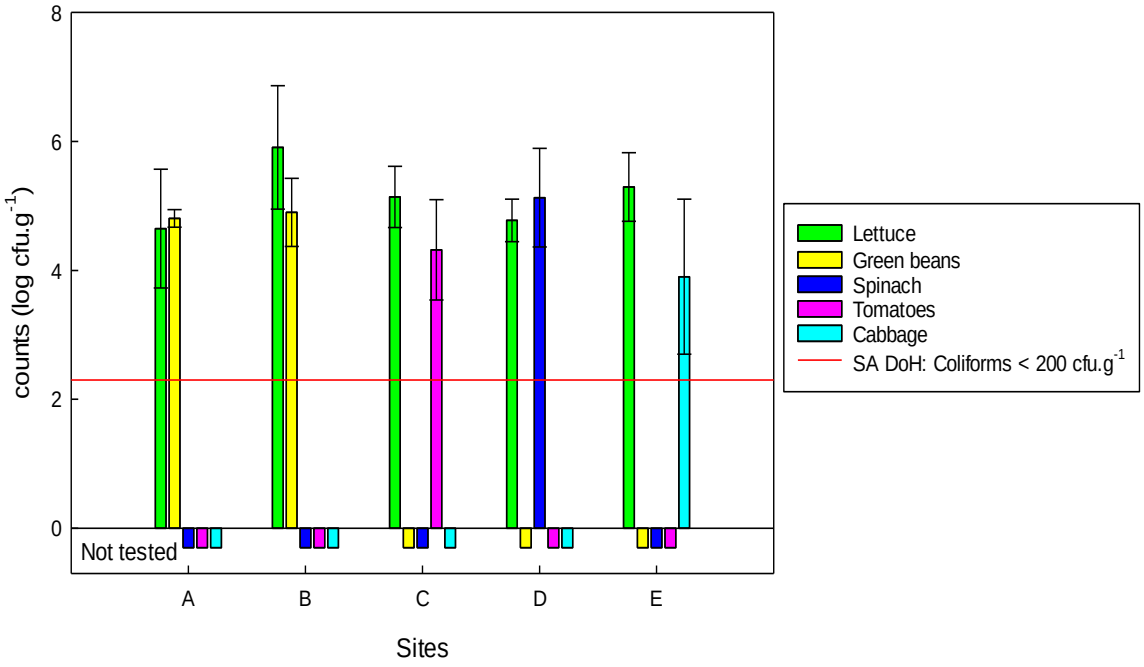

Figure 3.11 The total coliform counts of the selected fresh produce products sampled at sites A – E for repetition 3 of 3.

The colony counts of the lettuce samples from all sites collected during all repetitions were used to compare the overall microbiological quality at the sites. The same product was specifically sampled to be able to compare the sites to one another. The graph (Figure 3.12) gives an indication of the long-term quality of the lettuce sold at a specific site. This can be seen by comparing the quality of the fresh produce from the three repetitions.

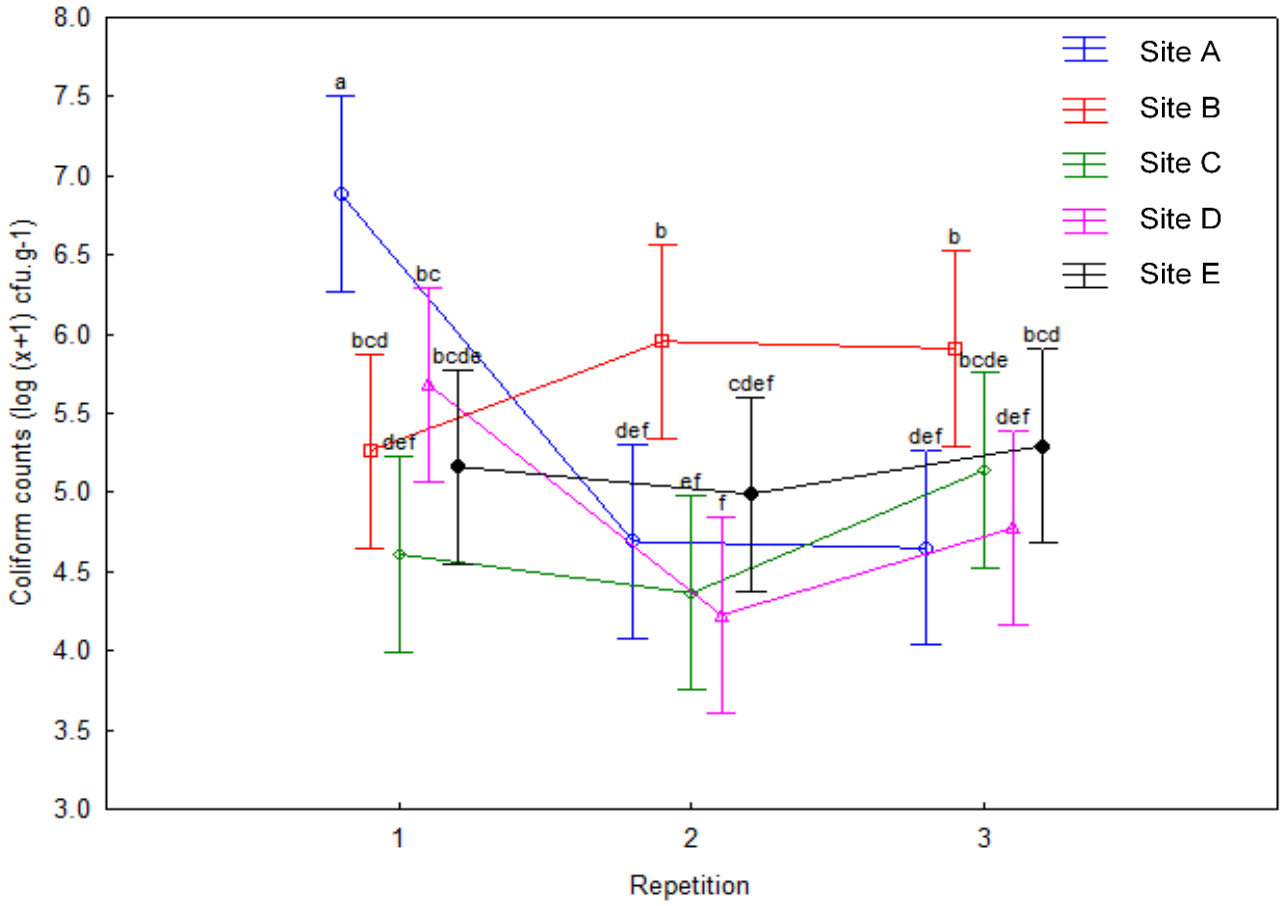

Figure 3.12 A general linear model illustrating significant differences for the coliform counts from lettuce at sites A – E. A significant difference (5%) is indicated with different letters.

According to the results presented in Figure 3.12, sites B, C and E were consistent in the quality of their lettuce products sold during repetition 1,2 and 3 because there were no significant differences between the coliform counts of the samples in the three repetitions. However, sites A and D showed larger variants in their lettuce's quality. The coliform counts from the lettuce samples collected during the first repetition of both sites A and D was significantly higher than the coliform counts from the lettuce samples collected in repetitions 2 and 3 (Figure 3.12). Therefore, it can be concluded that the quality of the fresh produce sold at site A & D was not consistent and therefore the consistency of the safety and quality of the fresh produce is questionable.

The indicator system for indicating possible enteric foodborne pathogens was determined by enumerating *Enterobacteriaceae*. *Enterobacteriaceae* were determined for produce samples from

all sites. Figure 3.13, 3.14 and 3.15 are the results of the *Enterobacteriaceae* counts for repetitions 1-3, respectively. The products not tested are indicated as a bar under the x-axis.

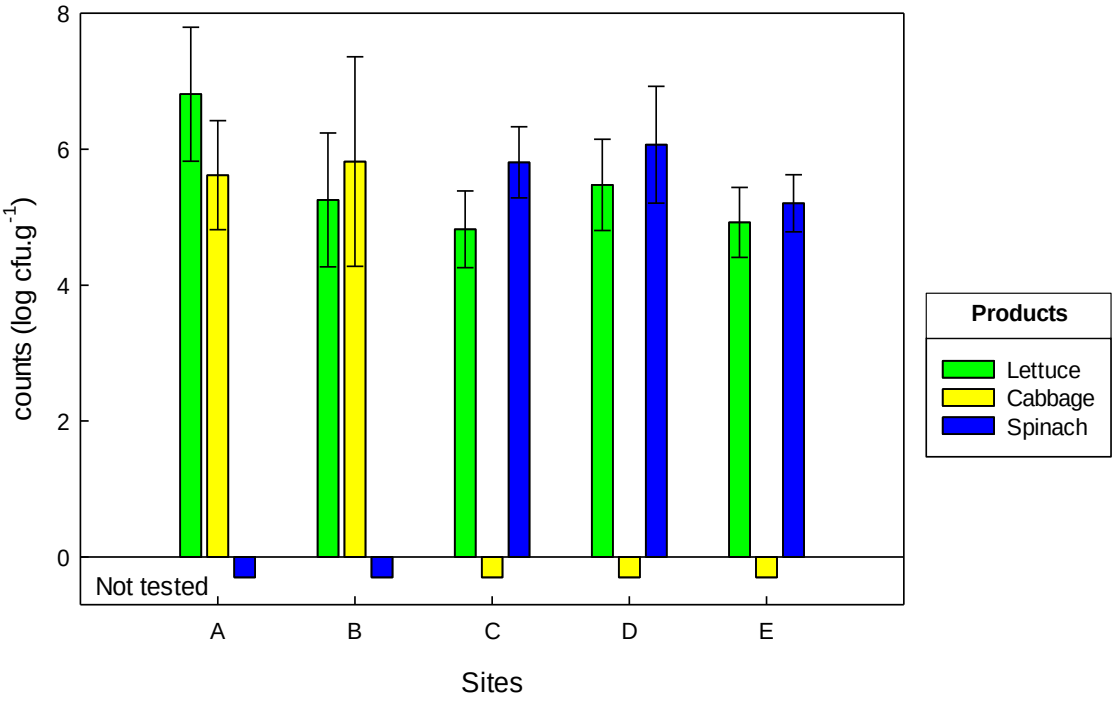

Figure 3.13 The total *Enterobacteriaceae* counts of the selected fresh produce products sampled at sites A – E for repetition 1 of 3.

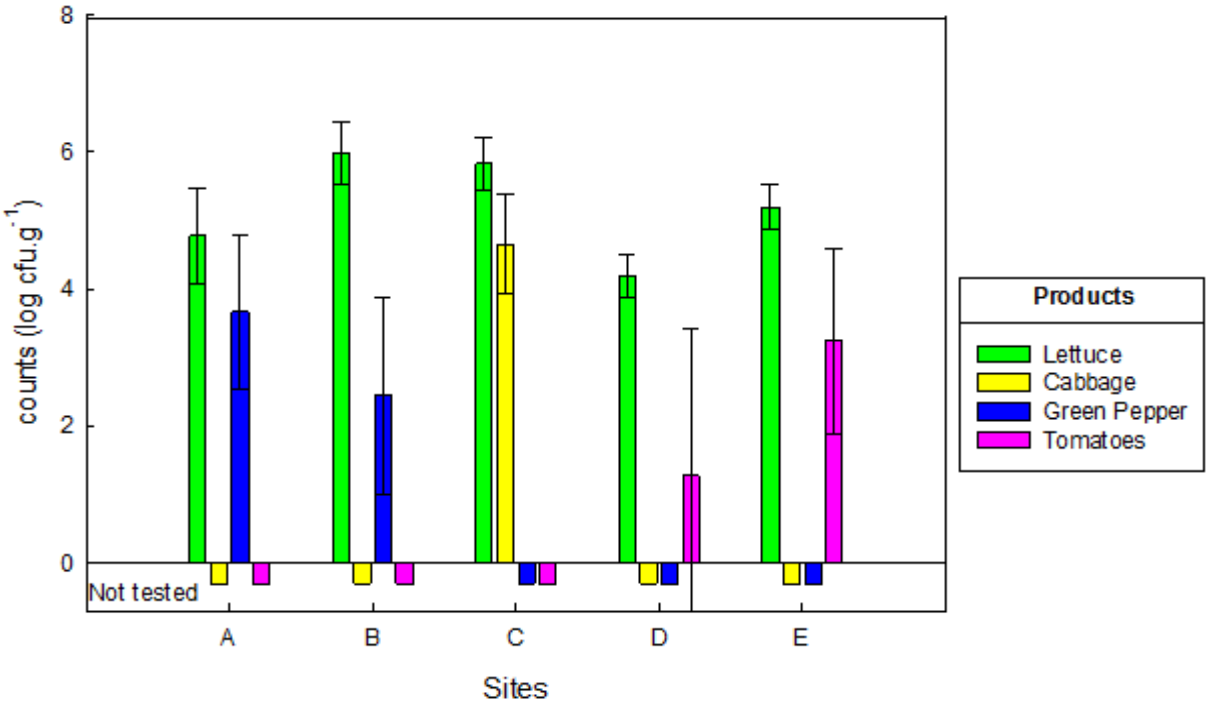

Figure 3.14 The total *Enterobacteriaceae* counts of the selected fresh produce products sampled at sites A – E for repetition 2 of 3.

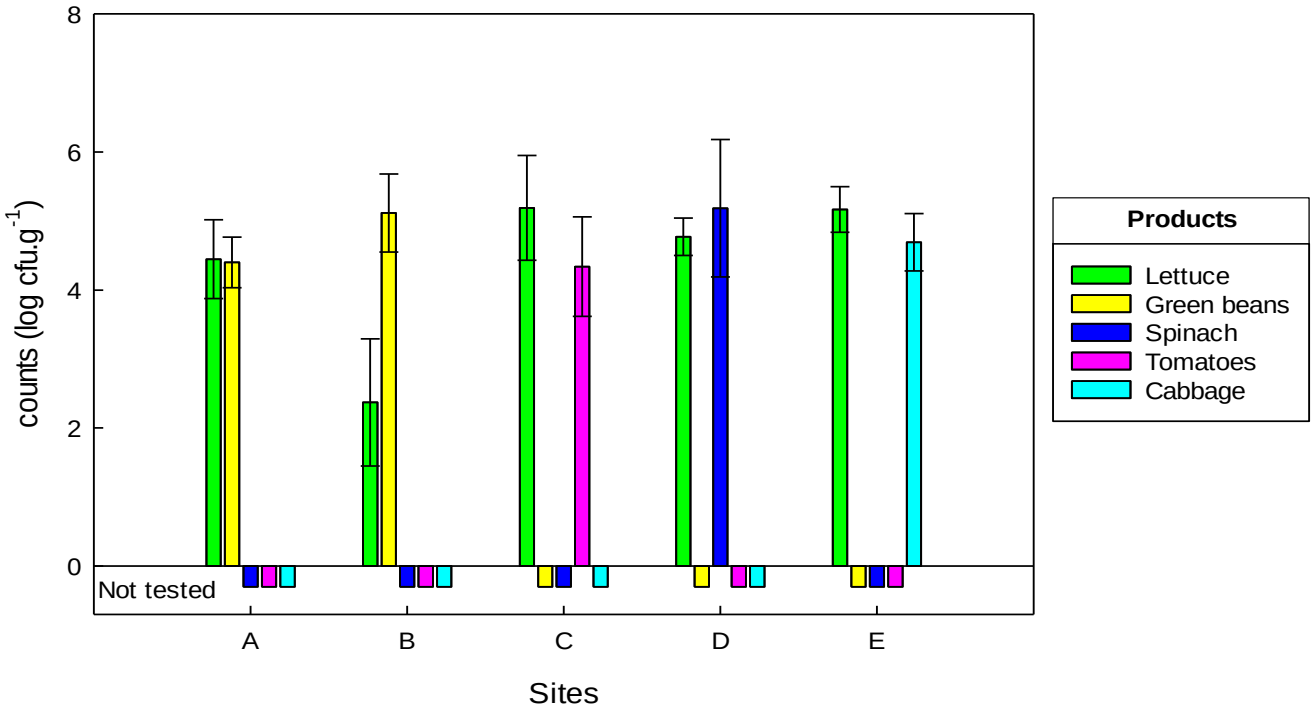

Figure 3.15 The total *Enterobacteriaceae* counts of the selected fresh produce products sampled at sites A – E for repetition 3 of 3.

The coliform and *Enterobacteriaceae* counts of repetition one was very similar to each other as seen in Figure 3.9 and Figure 3.13, respectively. There is no significant difference between the *Enterobacteriaceae* count and the coliform counts ($p > 0.05$). The samples in the first repetition did not contain of any *Enterobacteriaceae* bacteria that were not already classified as coliforms. This was the same for repetition 2 (Figure 3.14) and repetition 3 (Figure 3.15). There was no significant difference between the *Enterobacteriaceae* counts (Figure 3.13 – 3.15) and the coliform counts (Figure 3.9 – 3.11). In this study, the enumeration of coliforms was a sufficient enough indicator system to determine the general hygiene of fresh produce. However, this method of determining the general hygiene of a food product has limitations due to the fact that the coliform group does not give an indication of certain foodborne pathogens such as *Salmonella* spp. It is, therefore, useful to use the *Enterobacteriaceae* counts rather than the coliform counts because if pathogens such as *Salmonella* are present, there will be higher *Enterobacteriaceae* counts, giving an indication of potential presence of a pathogen. However, *Enterobacteriaceae* counts cannot be used as an indicator of food safety. High risk foodborne pathogens should still be tested for to ensure food safety. Hygiene indicator organisms such as *E. coli* will give a good indication of the handling practises of the product whereas *Enterobacteriaceae* will only give an indication of the overall microbiological quality of the product.

The *E. coli* levels in the fresh produce were determined for the selected fresh produce at all five sites. The same samples that were tested for coliforms and *Enterobacteriaceae* were also tested for

E. coli. Products not tested are indicated under the x-axis on the graph. Two microbiological limit guidelines are indicated on the graph (Figure 3.16). The European Food Safety Authority advises *an E. coli* limit between 100 – 1000 cfu.g^{-1} (EFSA, 2007). The second advised microbiological limit indicated on the graph is from the DoH and recommends a zero tolerance for *E. coli* (0 cfu.g^{-1}) (DoH, 2000).

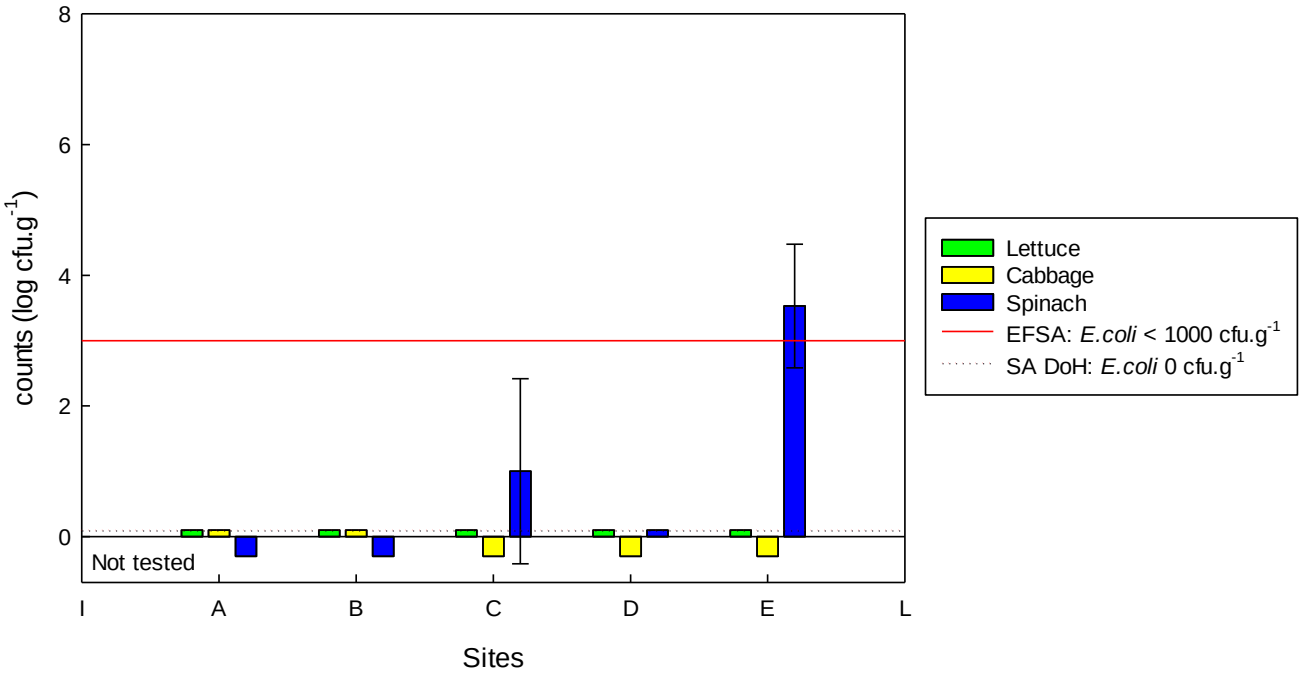

Figure 3.16 The total *E. coli* counts of the selected fresh produce products sampled at sites A – E for repetition 1 of 3.

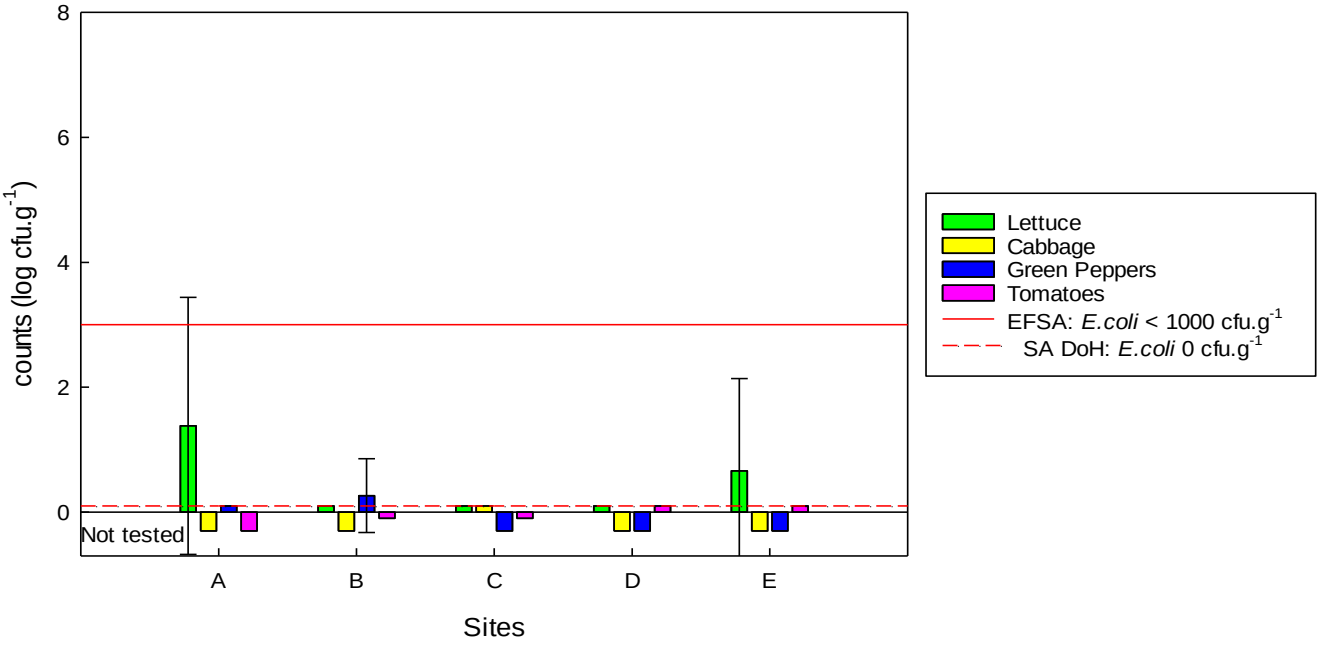

Figure 3.17 The total *E. coli* counts of the selected fresh produce products sampled at sites A – E for repetition 2 of 3.

Using South Africa's DoH's guidelines the results with the positive count for *E. coli* is regarded as unacceptable. In repetition 1 (Figure 16), conducted during the summer months between November and December only the spinach samples at sites C and E were positive for *E. coli* at an average level of 1.00 log cfu.g^{-1} and 3.53 log cfu.g^{-1}, respectively. Figure 3.16 only gives a representation of the average count for *E. coli* per site. In reality, only two spinach samples tested positive for *E. coli* at Site C, which resulted in a large standard deviation of 1.00 ± 1.42. However, for site E all five samples of spinach were positive for *E. coli,* although levels varied from 2.48 to 5.00 log cfu.g^{-1} (Table 3.6). These results could be as a result of a combination of poor handling practices, unprotected products with no packaging, poor storage or transport. In repetition 2, (Figure 3.17) the samples from sites A, B and E resulted in positive results for *E. coli*. In two cases only the lettuce samples tested positive for *E. coli,* with average levels at 1.38 ± 2.06 and 0.67 ± 1.48 cfu.g^{-1} respectively for site A and E. The presence of *E. coli* during repetition 2 also occurred sporadically. The lettuce samples from site A tested positive in only two lettuce samples and at Site E, only one lettuce sample tested positive for *E. coli*. A green pepper from site B also tested positive in one sample for *E. coli* at 1.32 log cfu.g^{-1} (Table 3.6).

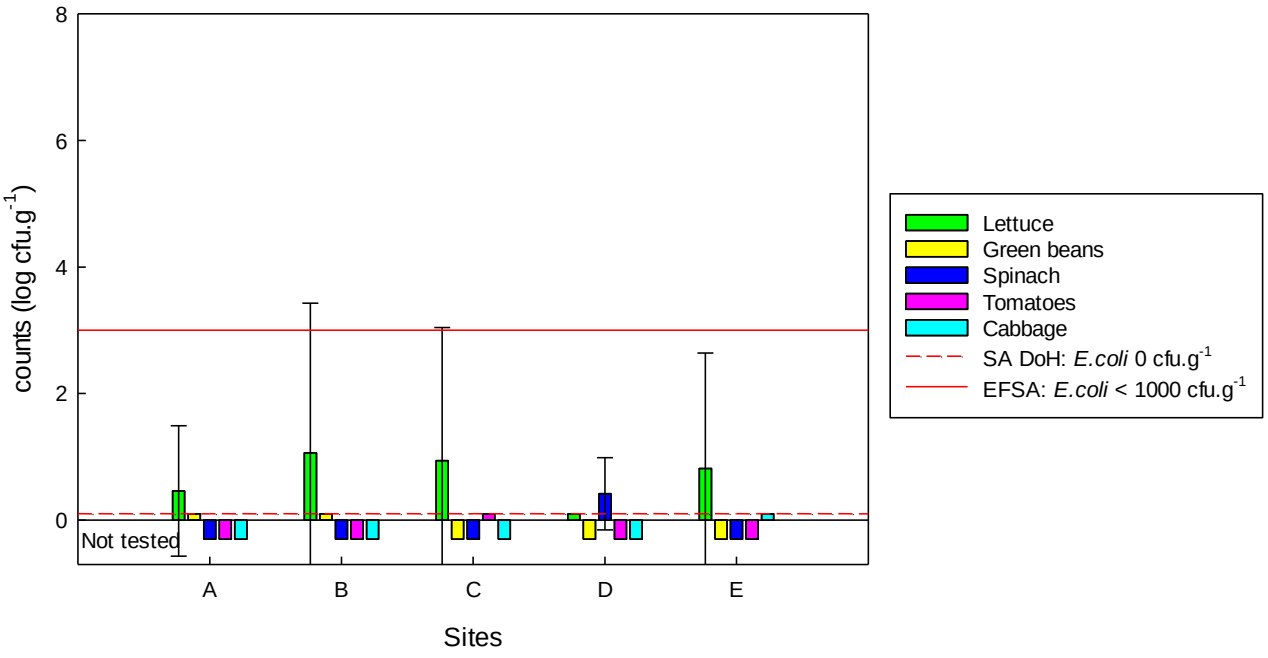

Figure 3.18 The total *E. coli* counts of the selected fresh produce products sampled at sites A – E for repetition 3 of 3.

Figure 3.18 gives a representation of the *E. coli* results of repetition 3 from sites A - E. The lettuce samples from this repetition tested positive for *E. coli* with the exception of site D. But, at site D, the spinach samples in turn tested positive for *E. coli* with average levels of 0.42 log cfu.g^{-1} ± 0.57. However, these average levels of 0.42 log cfu.g^{-1} ± 0.57 for spinach is a slight misrepresentation of the presence of *E. coli* on the spinach at site D. Only two of the five spinach samples contained *E. coli* (Table 3.6) and contained 1.04 log cfu.g^{-1} *E. coli* each. At site A, B, C and E, only one lettuce

sample tested positive for *E. coli* (Table 3.6). The *E. coli* counts in Figure 3.18 is therefore a slight misrepresentation of the actual *E. coli* levels of the results. The five subsamples are averaged to generate the graph and therefore does not give information about the individual *E. coli* counts for each product. It is often the case that only one or two of the products contain *E. coli* but not the rest. This illustrates the sporadically presence of *E. coli* on fresh produce. The true counts of the individual samples can be seen in Table 3.6.

Table 3.6 The results for product samples that tested positive for *E. coli* in repetition 1-3

Sampling date	Sampling site	*E. coli* counts log cfu. g^{-1}	Product	Sub sample nr
Repetition 1				
05/12/2017	C	3.00	Spinach	435
05/12/2017	C	2.00	Spinach	438
28/11/2017	E	2.48	Spinach	381
28/11/2017	E	3.70	Spinach	189
28/11/2017	E	3.00	Spinach	345
28/11/2017	E	5.00	Spinach	080
28/11/2017	E	3.48	Spinach	658
Repetition 2				
08/01/2018	A	4.60	Lettuce	416
08/01/2018	A	2.30	Lettuce	075
15/01/2018	B	1.32	Green Peppers	073
05/02/2018	E	3.30	Lettuce	857
Repetition 3				
12/03/2018	A	2.30	Lettuce	525
19/03/2018	B	5.30	Lettuce	175
03/04/2018	C	4.70	Lettuce	691
26/03/2018	D	1.04	Spinach	708
26/03/2018	D	1.04	Spinach	274
16/04/2018	E	4.08	Lettuce	399

One lettuce sample collected at Site A (repetition 2) tested positive for STEC and contained the *eae* and *stx* virulence genes. Figure 3.19 is a representation of a graph generated by the BAX® system showing the positive STEC result for lettuce sample 416 sampled at site A during repetition 2. It is unknown, however, what the amount of STEC was. However, the overall *E. coli* count for this particular lettuce sample at site A during repetition 2 was 4.6 log cfu.g^{-1}.

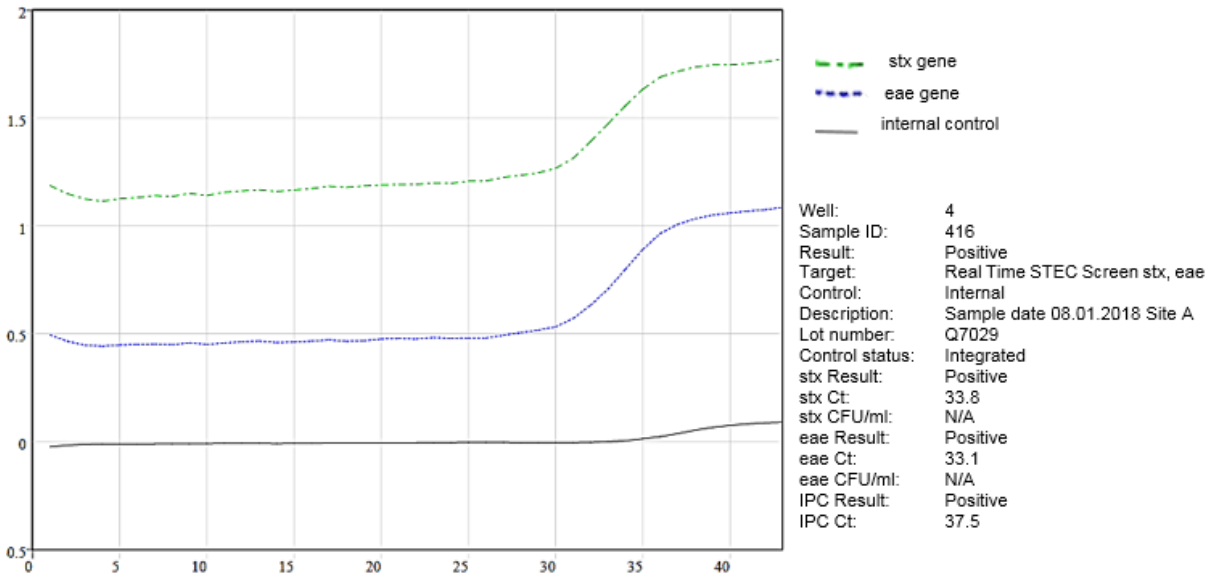

Figure 3.19 The realtime PCR graph clearly indicating the presence of the *eae* and *stx* gene for the lettuce sample 416 collected at Site A during repetition 2. The graph and result are generated by the BAX® system (Hygiena).

A similar study, to this study, was completed in the formal food sector in 2016 in the United States of America. Similar results were reported for the detection of *E. coli* in lettuce and spinach samples (Korir *et al.*, 2016). Korir *et al.* (2016) reported an 8.7% detection frequency for both lettuce and spinach, whereas this current study detected 10.6% *E. coli* prevalence for lettuce and spinach. The maximum *E. coli* levels in this study for lettuce were 1.38 log cfu.g^{-1} and spinach 3.53 cfu.g^{-1} compared to results of Korir *et al.* (2016), which reported *E. coli* levels for lettuce at 1.78 cfu.g^{-1} and spinach 1.30 cfu.g^{-1}. Maffei *et al.* (2013), a study conducted in Brazil, reported average *E. coli* levels of organically produced lettuce at 1.53 cfu.g^{-1} ± 0.54. Therefore, the South African informal market results, specifically in the Cape Town metropolitan area, are very similar to the results of the formal market in international countries.

Because *E. coli* is used as an indicator of hygiene practices, it is evident that the handling of fresh produce in the informal market in this study is poor. Informal traders do not have a direct and practical set of health requirements that they have to comply to in order to trade safe fresh produce or any other foodstuffs at informal trading markets or street vendors. Food handlers are expected to meet minimum safety requirements as required by customer and regulatory authorities. Food handlers in food handling areas, according to the SANS 10049:2012 (Food safety management – Requirements for prerequisite programmes) include farms, pack houses, fresh produce markets, manufacturing facilities, factory shops, catering units and kitchens, restaurants, butcheries, retailers, distribution centres and transporting vehicles. However, informal street vendor trading does not adhere to this standard – it is not practical in the informal sector. The fresh produce is typically handled, transported and stored at the informal trader's best knowledge with no formal handwashing

facilities or water infrastructure available. Microbiological contamination is expected due to the transfer of handler's hands. The fresh produce, for example the tomatoes and green beans, are packed by hand at the informal vendor and are thus handled extensively and individually. The site is secured overnight but is not enclosed and is therefore exposed to dust from the environment including street traffic, passing travellers and street sweeping which may settle on the fresh produce. Pests such as rodents and insects may also still have access to produce.

The detection of the selected foodborne pathogens including *Salmonella* spp., STEC and *Listeria monocytogenes* was completed for all fresh produce samples and the results are presented in the table 3.7.

Table 3.7 A summary of all the foodborne pathogen results obtained during repetition 1-3 for all the sites tested (A – E)

Foodborne pathogen tested	**Results for all products at site A - E**
Repetition 1	(n=50)
Salmonella spp.	Negative 0%
STEC	Negative 0%
Listeria monocytogenes	Negative 0%
Repetition 2	(n=50)
Salmonella spp.	Negative 0%
STEC	Negative 0%
Listeria monocytogenes	Negative 0%
Repetition 3	(n=50)
Salmonella spp.	Negative 0%
STEC	**Positive 2%**
Listeria monocytogenes	Negative 0%

There were no pathogens detected (Table 3.7) in any of the samples at any of the sites during repetition 1 – 3 with only one exception: STEC was detected in one lettuce sample isolated at site B during repetition 2.

3.4.2.3 Food safety of the selected fresh produce

The average coliform counts of five samples of one product was calculated and plotted against the different products (cabbage, lettuce, spinach, green peppers, tomatoes, green beans) to determine whether there is a significant difference between different products. The letters on the graph indicate significant differences.

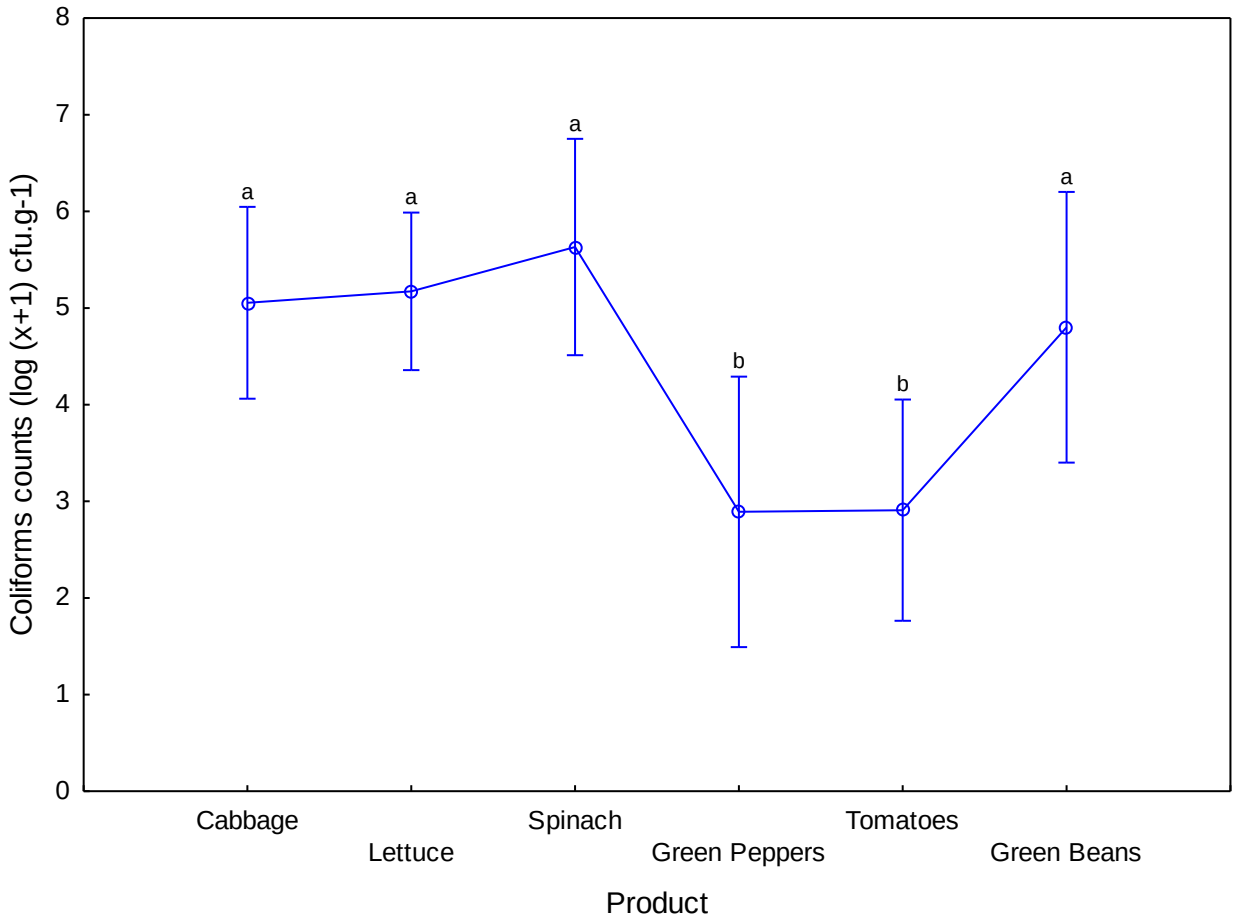

Figure 3.20 Shows the significant differences between the coliform count of all the products tested in repetition 1 – 3 indicating the similarities between different products.

Figure 3.20 shows the average coliforms counts of the respective products over all the sites and repetitions. The data presented in Figure 3.20 indicates significant differences between some products in terms of the overall hygiene indication (coliform counts). There was no significant difference between the lettuce and cabbage ($p = 0.83$), lettuce and spinach ($p = 0.46$) or lettuce and green beans ($p = 0.61$). Therefore, it is conclusive that there were no difference in the general hygiene of lettuce, cabbage, spinach or green beans. An interesting fact to note is that spinach naturally spoils very quickly because of the nature of the crop (Escalona *et al.*, 2010). Therefore, a higher coliform count was expected. However, this was not the case. In practice, spinach was purchased freshly every day and was sold within the same day the spinach was sourced in contrast with the other products that were bought in bigger quantities and kept at the informal vendors for a longer period. It was unknown how long the products were stored at the vendor before being sold. The two products that did show some deviation from the rest of the products are green peppers and tomatoes with a significant difference between lettuce and green peppers ($p = 0.009$) and lettuce and tomatoes ($p = 0.004$). A possible explanation for the lower coliform counts in tomatoes could be the presence of a lower pH of the vegetable. Bacteria do not survive well in acidic conditions and the low pH inhibits the growth of bacteria (Chikazunga *et al.*, 2008; Forsythe, 2010) . The antimicrobial

agent used in agriculture for fungicides/pesticides can also affect the microbiological load (Olaimat and Holley, 2012).

3.5 CONCLUSIONS

During the evaluation of the overall microbiological safety of fresh produce in the informal sector, investigated in this study, it was found that all the products at the respective sites exceeded the microbiological limit for coliforms set out by South Africa's DoH guidelines. Green peppers and tomatoes were the exception and overall were within the DoH limit. The low pH of the tomatoes and possible use of pesticides on green peppers, that also control the overall microbiological load, could possibly be contributors of the low coliform and *Enterobacteriaceae* counts for tomatoes and green peppers (Olaimat and Holley, 2012). Overall the spinach, lettuce, cabbage and green beans had no significant difference in terms of their coliform counts. But green peppers and tomatoes significantly differed from the rest of the products. The overall microbiological quality of the green peppers and tomatoes were better than the lettuce, spinach, cabbage and green beans when comparing the coliform and *Enterobacteriaceae* counts of the products.

The overall microbiological counts of site B were the least acceptable. Along with the pathogen detection results, site B turned out to be the site with the lowest microbiological safety. However, site B was not significantly different to any other site in terms of the overall microbiological counts. But site B was the only site where one lettuce product was positive for STEC. No pathogens were detected at any other sites. The prevalence of *E. coli* counts increased from repetition 1 to 3. Lettuce and spinach were the only two products that tested positive for *E. coli*. During repetition 1, only spinach contained *E. coli* at two sites (Site C and E). During repetition 2, spinach and lettuce contained *E. coli* at three sites (site A, B and E). During the third repetition, the lettuce and spinach at four sites contained *E. coli*. The increasing prevalence of *E. coli* could be due to the drought that Cape Town has experienced during the time of sampling. The water quality decreases significantly during climatic changes, especially droughts, increasing the risk of microbiological contamination (Liu *et al.*, 2013).

The constant high coliform counts throughout all the sites and products could be due to pre-harvest contamination factors such as contaminated irrigation water which has a large impact on the final produce (Liu *et al.*, 2013; Romanis, 2013). It can be speculated that the coliform contamination is from pre-harvest factors because the coliforms counts for all the sites contained the same average coliform counts at 5 log cfu.g^{-1}. The post-harvest contamination would not have had such a large impact on the elevated coliform levels because all products from all the sites were handled, transported and stored differently.

In South Africa the fresh produce of the highest quality is sold in the export market (SADC, 2007). After the export market demand is satisfied, the best produce is sold in the formal retailers, restaurants and distribution centres. The informal market is the last location in the supply chain that

receives fresh produce stock. In the informal market, the produce is handled, transported and stored in an uncontrolled environment. Therefore, the informal market is expected to sell the worst-case scenario product in terms of the microbiological safety of the product. However, it is seen from previous studies, and it is confirmed in this study, that pathogens are not so commonly found on fresh produce (van Dyk *et al.*, 2016; Jongman and Korsten, 2017; Du Plessis *et al.*, 2017). Direct contamination, ideal growth temperatures, fresh produce that is not washed or washed with contaminated water are a few post-harvest events and conditions that should take place in order to find fresh produce contaminated with pathogens (Gil *et al.*, 2015). Never the less, fresh produce is a high-risk food product and pre- and post-harvest prosses must be controlled.

The *E. coli* and coliform counts of the fresh produce correspond to international countries' formal market counts with the exception of spinach, for which the South African counts were slightly higher (Korir *et al.*, 2016; Maffei *et al.*, 2013). Although there is no health regulation in SA for informal trading, that manage the food safety risk, the coliform counts still corresponds to the fresh produce from the South African formal market (Jongman and Korsten, 2016). Overall, during the time of this study, the microbiological safety of fresh produce in the informal sector is average, being over the South African microbiological recommended limit.

There is a natural amount of *Enterobacteriaceae* present on fresh produce and can be expected (Little *et al.*, 1999). The product is not sterile and is not heat processed before consumption and therefore there will be a natural amount of coliforms/*Enterobacteriaceae* present. *Enterobacteriaceae* should therefore not be used as the only indicator of good hygiene practices. Instead, faecal organisms such as *E. coli* that have a direct correlation to poor handling practises should be used (Little *et al.*, 1999). The risk analysis for food safety is not complete by just enumerating the *Enterobacteriaceae* or coliforms as hygiene indicator organisms. The most prevalent and high-risk pathogens in fresh produce should still be tested which includes *Salmonella* and *E. coli* (STEC).

The overall microbiological safety of fresh produce in the informal retailers in the Cape Town Metropolitan area in South Africa was of unacceptable quality when only comparing the coliform counts to the limit advised by the DoH. However, the fresh produce contained no pathogens with, exception of one lettuce sample containing STEC. The presence of *E. coli* occurring sporadically suggests poor handling practices. The immune response of an individual also plays a role when consuming fresh produce contaminated with *E. coli*. An individual will most likely only be affected severely when the individual's immune response is low and when the pathogenicity of the consumed *E. coli* is high. Regardless of the high coliform counts and the presence of *E. coli* in sporadically occurring events, there is no evidence supporting that fresh produce tested being unsafe for consumption.

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CHAPTER 4

THE PRESENCE OF EXTENDED SPECTRUM BETA-LACTAMASE-PRODUCING ORGANISMS IN FRESH PRODUCE AND THE DETERMINATION OF THEIR ANTIBIOTIC RESISTANCE PROFILES

4.1. SUMMARY

The purpose of this study was to determine the presence and prevalence of Extended Spectrum β-Lactamase (ESBL) producers in fresh produce. Fresh produce (lettuce, cabbage, spinach, tomatoes, green beans and green peppers) was sampled at five selected informal vendors. The presence of ESBL producers was screened using ESBL ChromID agar (Biomerieux). After the ESBL producers were isolated, the isolates were subjected to phenotypical testing (combination disk diffusion test) to confirm their ESBL status. The isolates were further subjected to molecular confirmation (Multiplex PCR), identifying the three most prevalent genes in ESBL producers namely *bla*$_{SHV}$, *bla*$_{CTX-M,}$ *and bla*$_{TEM}$. The antibiotic resistance profiles of the isolates were determined by subjecting the isolates to antibiotics in eight different antibiotic classes. Of the 416 presumptive positive ESBL producers that were isolated, 158 isolates were identified using the MALDI-ToF technique. Thirteen (8.2%) of the identified isolates were confirmed from the family *Enterobacteriaceae*: *Klebsiella pneumonia* (4/13), *Enterobacter* (8/13) and *E. coli* (1/13). The rest of the isolates consisted of mostly *Pseudomonas* (129/158), *Achromobacter* (5/158), *Stenotrophomonas maltophilia* (2/158) and 8/158 that were unidentified. Of the 13 confirmed to be *Enterobacteriaceae*, seven isolates were confirmed as ESBL producing *Enterobacteriaceae*. The *bla*$_{SHV}$, *bla*$_{CTX-M,}$ *and bla*$_{TEM}$ genes were detected in some of the confirmed ESBL producing *Enterobacteriaceae* suggesting that other mechanisms causing ESBL like mechanisms are also present. All the confirmed ESBL producing *Enterobacteriaceae* (7) were also multidrug resistant, being resistant to at least Ampicillin (Penicillin's), Cloxacillin (Cephalosporins) and Ciprofloxacin (Fluoroquinolones). This study provided a snapshot of the current situation regarding the presence and prevalence of ESBL producing *Enterobacteriaceae* in fresh produce in the informal market in the Cape Town metropolitan area, South Africa. Although ESBL producing *Enterobacteriaceae* were present in the tested fresh produce products, the prevalence was relatively low (4%, 7/158). Multidrug resistance amongst ESBL producing *Enterobacteriaceae* was extremely high (100%, 7/7), however, it is unknown whether these organisms carry their resistance genes on the plasmid DNA which would increase the dissemination of resistant genes in the environment. It is therefore important to acknowledge the fact that ESBL producing *Enterobacteriaceae* are present in fresh produce and that these organisms

might have the ability to transfer their resistant genes to opportunistic foodborne pathogens, increasing the current antibiotic resistance problem and be a direct health threat to consumers.

4.2. INTRODUCTION

Since the discovery of antibiotics in the 1900's, life-threatening infections became an issue of the past. However, only two years after the discovery, the first antibiotic resistance was observed (Shlaes *et al.*, 2004). Antibiotic resistance is now one of the most serious global medical challenges (Ferri *et al.*, 2017). The Extended Spectrum β-Lactamase (ESBL) producing *Enterobacteriaceae* is one of the top six main antibiotic resistance related health risks in the world (Chong *et al.*, 2018). ESBL producing *Enterobacteriaceae* are organisms that carry genes that enable the bacteria to produce enzymes that chemically inactivate antibiotics when given to a patient (Paterson & Bonomo, 2005). The third generation cephalosporins are often the antibiotics that are used as last resort to treat a patient against life-threatening infections. ESBL producing *Enterobacteriaceae* are becoming more and more resistant to third generation Cephalosporins and sharing their genetic material amongst other bacteria in the same environment, increasing the antibiotic resistance problem (Ferri *et al.*, 2017). According to the Centre for Disease Control (CDC), ESBL producing *Enterobacteriaceae* are the cause of approximately 9 000 infections per year in the USA and responsible for 600 deaths (CDC, 2013). ESBL producing *Enterobacteriaceae* are called "the nightmare bacteria".

The presence of antibiotic resistant bacteria has been observed worldwide in surface water, which is often used as irrigation water for fresh produce (Blaak *et al.*, 2015). The South African surface waters used for irrigational purposes are seriously compromised by sewage from informal settlements, waste from animal production, inadequate sanitation and from the mining sector (Romanis, 2013). These microbiological contaminants end up in the rivers and have the opportunity to mix with diluted antibiotics that also end up in the rivers as a result of overuse of antibiotics in society. This is where environmental bacteria gain the opportunity to either develop resistant genes or to transfer the genes from one organism to another (Ferri *et al.*, 2017). Antibiotic resistant bacteria can also transfer their genes to pathogenic organisms (Ferri *et al.*, 2017).

Previous studies have shown a prevalence of 5.2% of ESBL producing *Enterobacteriaceae* (3rd-generation resistant *Enterobacteriaceae*) in retail vegetables produced in the Netherlands (Van Hoek *et al.*, 2015). According to Cantón *et al.* (2008) the prevalence of ESBL producers in Europe is higher than in the USA but lower in South America and Asia. The spread of epidemic plasmids have been responsible for the increase in ESBL producers in the European countries according to Cantón *et al.* (2008). A study by Nüesch-Inderbinen *et al.* (2015) reported that ESBL producers are mostly found in the irrigation water used for fresh produce rather than on the fresh produce itself. Most of the ESBL producers found were also multidrug resistant. With the high concern expressed by the CDC in regard to ESBL producers causing many serious infections, the importance of the

surveillance of fresh produce (that is consumed raw) as a source of ESBL producing *Enterobacteriaceae* is evident. Currently, to the best of the author's knowledge, there is no information available on the presence of ESBL producers on fresh produce in South Africa.

Antibiotic resistant genes can be spread by various mechanisms including conjugation, transformation and transduction, with conjugation being the most common mechanism (Ferri *et al.*, 2017). It involves plasmid DNA which replicates independently of chromosomal DNA. When two bacteria proliferate in the same environment, be it in the human or animal gut, water or soil, it is possible for the plasmid DNA containing the resistant gene to duplicate and be transferred to other bacteria through a bridge like structure (pilus) (Carattoli & Elena, 2009). The next time the bacteria are exposed to the antibiotic corresponding to the new resistant gene, the bacteria now have the ability to produce an enzyme that chemically inactivates the antibiotic agent, making the antibiotic ineffective (Brunton *et al.*, 2011). Other resistance mechanisms can also occur including mutations and the use of efflux pumps that actively pump the antibiotic agent out of the cell before damage can be done (Livermore *et al.*, 2002).

ESBL producing organisms, in general, have the capability of hydrolysing penicillins, cephalosporins and monobactam aztreonam antibiotics, but not cephamycins or carbapenems (Perez *et al.*, 2007). In addition, ESBL producers, especially TEM and SHV family derivatives, are inhibited by β-lactamase inhibitors such as clavulanic acid (Perez *et al.*, 2007). This unique property of the ESBL producers makes it possible to distinguish and identify ESBL producers via phenotypical testing (EUCAST, 2017a). Testing involves the use of either Ceftazidime or Cefotaxime and Cefepime in combination with clavulanic acid according to the combination disk diffusion method (EUCAST, 2017a). Bacteria are confirmed as ESBL producers if the inhibition zone diameter is ≥ 5 mm larger with clavulanic acid than without.

The aim of this study was to determine the presence and prevalence of Extended Spectrum β-Lactamase producing organisms as well as their broader antibiotic resistance profiles in fresh produce sold at informal markets in South Africa. This was achieved by initially screening for ESBL producing *Enterobacteriaceae* using ESBL ChromID agar (Biomerieux). The ESBL status was confirmed by using phenotypical confirmation (antibiotic disk diffusion method) and a molecular method (Polymerase chain reaction (PCR) confirmation and electrophoresis). The antibiotic resistance profiles of the ESBL producers was determined by exposing the organisms to eight antibiotics in different classes to confirm the possibility of multidrug resistance. A bacterium is classified as multidrug-resistant if it is resistant to three or more agents in different classes of antibiotics (Doyle *et al.*, 2013).

4.3. MATERIALS AND METHODS

Screening for ESBL Enterobacteriaceae

For the screening of ESBL *Enterobacteriaceae*, a method adapted from Zurfluh *et al.* (2015) was used. A 1 mL inoculation was made into EE broth (Merck, South Africa) from macerated 25 g sample in 225 mL BPW which was previously incubated at 37˚C for 24 hours. The inoculated EE broth was incubated for 24 hours at 37˚C. After incubation, the broth was streaked out onto ESBL ChromID agar (Biomerioux, South Africa) and incubated for 24 hours at 37˚C. The presence of growth indicated presumptive positive ESBL producing *Enterobacteriaceae*. The colour reactions gave an indication of the species of *Enterobacteriaceae* present. A pink or blue colony can be a presumptive ESBL producing *E. coli* whereas green can indicate the presence of *Klebsiella, Enterobacter, Serratia* or *Citrobacter*. Colourless colonies with a brown halo give an indication of *Proteus, Morganella* or *Providencia* and colonies without a brown halo are considered indicative *of Salmonella* or *Acinetobacter*. Colonies that grew on the ESBL ChromID agar were further isolated by transferring colonies of different colours from the chromogenic agar to VRBG agar (Merck, South Africa). Each isolate was given a unique number and stored in 40% v/v glycerol at -80˚C until further analysis.

Identification of ESBL Enterobacteriaceae using MALDI-ToF

After the isolation of presumptive positive ESBL producing *Enterobacteriaceae*, the isolates were purified and prepared for species identification. The identities of the isolates were confirmed using the MicroFlex LT Matrix-assisted laser desorption/ionisation time of flight (MALDI-ToF) mass spectrometer (Bruker Daltonics, Germany). The MALDI Biotyper 3.0 software (Bruker Daltonics, Germany) was used to compare the spectra to a reference spectrum database to determine the identity of each tested isolate. The system generates a logarithmic score that correlates to the similarity of the spectra and is interpreted according to the manufacturer's guidelines. A high log value generated (≥ 2.300) indicates a high level of confidence in species identification. Intermediate log value (≥ 2.000) indicates probable species identification whereas a value between 1.700 and 1.999 only provides genus identification. A log value < 1.700 does not generate identification. This could either be due to the presence of a mixed culture, or the isolated provided for identification is not captured in the present database and therefore, cannot be identified (Z. Zulu, 2018, Laboratory analysist, University of Pretoria, Pretoria, South Africa, personal communication, 15 June.).

Confirmation of ESBL Enterobacteriaceae

Phenotypical confirmation: Antimicrobial susceptibility testing

The EUCAST combination disk diffusion method was used to confirm the ESBL status of the isolates (EUCAST, 2017b). A selection of the presumptive positive isolates was subjected to confirmation testing after MALDI-ToF identification. The isolates were classified as group 1 or 2 according to the EUCAST guidelines presented in Figure 4.1. The isolates were confirmed as ESBL producing

Enterobacteriaceae using a respective agent (Ceftazidime, Cefotaxime or Cefepime) according to the group classification. Ceftazidime and Cefotaxime were used in combination with, and without, clavulanic acid for isolates such as *E. coli* and *Klebsiella* (group 1). Isolates such as *Enterobacter* that possibly carried an inducible chromosomal AmpC gene was confirmed using Cefepime with, and without, the combination of clavulanic acid (EUCAST, 2017a). The isolate subjected to the combination disk is confirmed positive if the inhibition zone diameter is ≥ 5 mm larger with clavulanic acid than without.

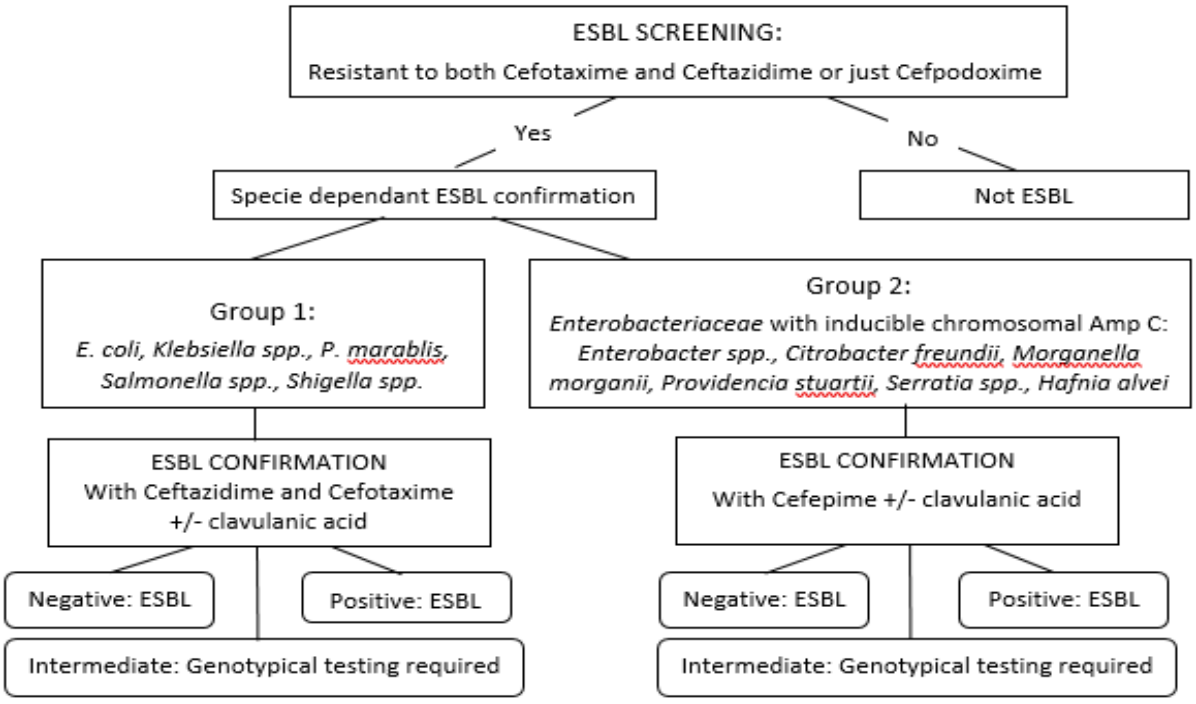

Figure 4.21 A decision tree for phenotypic detection of ESBL's adapted from EUCAST guidelines (EUCAST, 2017a).

Molecular confirmation: Multiplex polymerase chain reaction & Electrophoresis

DNA extraction:

Microbial DNA was extracted using the crude extraction boiling method (Altalhi & Hassan, 2009). A pure overnight culture was streaked onto nutrient agar. Single colonies were picked using a sterile inoculation loop and suspended into 100 µL of sterile, RNase free water (VWR Life Science, USA). The isolate was boiled at 100˚C for 13 minutes using a thermocycler (Bio-Rad, South Africa) and centrifuged for 15 min at 5 000 x g. The cell components (pellet) was discarded and the DNA containing supernatant was transferred to a new tube and stored at – 20˚C until further use.

The DNA concentration of the extract was determined using the Nanodrop Spectrophotometer ND 1000 and the corresponding ND-1000 V 3.8.1 software (Nanodrop technologies Inc, USA). The Nanodrop measures the absorbance of molecules that absorb light in the Ultraviolet wavelength region. Nucleotides, RNA, single-stranded DNA (ssDNA) and double-

stranded DNA (dsDNA) will absorb in the range of 260 nm. An absorbance value between 1.8 and 2.0 for the 260/280 nm ration is generally accepted as “pure” DNA. Ration values higher or lower indicate the presence of other molecules including proteins, phenols, salts or other cell components that absorb light at different regions and will influence the 260/280 ratio. The concentration of DNA is calculated by the ND-1000 V 3.8.1 software and is reported in ng.μL^{-1} (A. Vorster, 2018, Laboratory analysist, Central analytical facilities, Stellenbosch, South Africa, personal communication, 6 September.)

Multiplex Polymerase Chain Reaction (PCR):

The multiplex PCR was performed using the method described by Monstein *et al.* (2007) for the detection of *bla*$_{CTX-M}$, *bla*$_{SHV}$ and *bla*$_{TEM}$. The primer sets used for the target genes are listed in Table 4.1. The PCR amplification reaction was performed in 12.5 μL volumes. The KAPA2G Fast Multiplex kit (Kapabiosystems, South Africa) was used and consisted of KAPA2G Fast HotStart DNA Polymerase, 0.2 mM of each dNTP and 3 mM $MgCl_2$ (at 1X). The multiplex amplification reaction consists of 6.25 μL of the KAPA2G Fast Multiplex kit, 1.25 μL of each of the primers sets (3.75 μL total) at a 0.2 mM concentration, 1.5 μL of sterile, RNA free water (VWR Life Science, USA) and 1 μL of previously extracted DNA. A positive and negative control was included in all experiments. The negative control consists of 1 μL of sterile water. The positive control consists of a 50:50 combination of *E. coli* ATCC 35218 containing *bla*$_{TEM}$ and *Klebsiella pneumonia* previously isolated containing *bla*$_{CTX-M}$ and *bla*$_{SHV}$ (D. Rip, 2017, Researcher, Department of Food Science, Stellenbosch, South Africa, personal communication, June). The PCR mixture was subjected to the following thermocycling conditions using Gene Technologies G-Storm GS482 Thermal cycler (Vacutec, South Africa): 3 min initial denaturation at 95˚C followed by 30 repeat cycles of denaturation at 95˚C for 30 s, primer annealing at 60˚C for 30 s and extension at 72˚C for 30 s. Final extension took place at 72˚C for 10 min. The PCR products were then cooled down to 4˚C and then stored at -18˚C until the PCR products were subjected to electrophoresis for visualisation.

Table 4.1 Primer sequences for the amplification of *bla*$_{CTX-M}$, *bla*$_{SHV}$ and *bla*$_{TEM}$ target genes

Target gene	Primer sequence	Amplicon Size (bp)	Reference
bla$_{TEM}$	F: 5’-TCGCCGCATACACTATTCTCAGAATGA -3’ R: 5’- ACGCTACCGGCTCCAGATTTAT-3’	445	Paterson *et al.,* 2003
bla$_{SHV}$	F: 5’-ATGCGTTATATTCGCCTGTG-3’ R: 5’-TGCTTTGTTATTCGGGCCAA -3’	747	Monstein *et al.,* 2007
bla$_{CTX-M}$	F:5’-ATGTGCAGYACCAGTAARGTKATGGC -3’ R: 5’-TGGGTRAARTARGTSACCAGAAYCAGCGG -3’	593	Boyd *et al.,* 2004

Electrophoresis visualisation:

Preliminary work was completed using a 1.2% agarose gel containing 10 000 x EZ-Vision® Bluelight DNA dye (VWR Life Science, USA) to visualise the DNA fragments using the Bio-Rad Gel doc XR+ System (Bio-Rad, South Africa) in combination with the Image Lab software version 5.2.1. The size of the PCR amplicons was determined by comparing to a 100bp DNA marker (Promega, USA). The conditions for DNA fragment separation was 70 V for 100 minutes.

Microfluidic electrophoresis

Experimental work was completed using the LabChip GX II Touch microfluidic electrophoresis instrument (PerkinElmer, South Africa). The HT DNA 12K Reagent Kit (PerkinElmer, South Africa) was used with the DNA Extended range LabChip (PerkinElmer, South Africa). The diluted PCR products (10 µL of a 1:99 dilution) was assayed on the LabChip GX II Touch microfluidic electrophoreses instrument using the reagent kit and chip according to the manufacturers protocol along with the LabChip GX reviewer version 5.3.2115.0 software (PerkinElmer, South Africa) (A. Vorster, 2018, Laboratory analysist, Central analytical facilities, Stellenbosch, South Africa, personal communication, 19 September.).

The LabChip GX Touch instrument makes use of a chip which is loaded with all the needed reagents as well as the PCR products. These wells are connected with microfluidic microchannels that allow access to the different agents when needed. The PCR products are injected and move through a separation channel one by one and are detected via laser-induced fluorescence when the DNA fragment moves past the detection point. The individual DNA samples (PCR products) are separated electrophoretically based on the fragment size. The sizing and concentration of each band are determined using an internal and external ladder to align the individually analysed fragments. The system generates an electropherogram from which a simulated gel was visualised (A. Vorster, 2018, Laboratory analysist, Central analytical facilities, Stellenbosch, South Africa, personal communication, 19 September.).

Antibiotic resistance profiles

The isolates were exposed to a range of antibiotics from different antibiotic classes using the EUCAST disk diffusion method (EUCAST, 2017b). Mueller-Hinton agar for non-fastidious organisms was used. A fresh culture suspension was prepared using 18 - 24 hour colonies cultured on Nutrient agar (Merck, South Africa) at 37˚C and was compared to a 0.5 McFarland standard. The Mueller Hinton agar was inoculated using a sterile cotton swab. To avoid over-inoculation, excess fluid was removed by pressing against the walls of the tube and turning the swab. The Mueller-Hinton plates were inoculated by moving the dipped swab in narrow movements in three directions (horizontal, vertical and diagonal) to cover the complete surface of the agar plate. Seven antibiotic discs were transferred to the agar by either using an antimicrobial disk dispenser (Thermo Scientific, South Africa) or a sterilised pinset. All tests were done in duplicate. The plates were inverted and incubated

for 18 hours at 37˚C. The 15-15-15-minute rule was followed during the experimental procedure (EUCAST, 2017b). It entailed to use the pure (0.5 McFarland standard) culture suspension within 15 minutes of preparation, to apply the antibiotic disks within 15 minutes of the inoculated plates and to incubate the plates within 15 minutes of disk application. The antibiotics agents are listed in Table 4.2. A positive control organism (*E. coli* ATCC 35218) was included in every experiment. The results were gathered by measuring the zone diameter in mm using a calliper. The zone diameter was compared to standard breakpoint values for *Enterobacteriaceae* and specific agents published in the EUCAST tables (EUCAST, 2018). The breakpoints are summarised in Table 4.3. An organism is classified as multi-drug resistant when resistant to three or more antibiotics in three different classes (Doyle *et al.*, 2013).

Table 4.2 A summary of the antibiotic agents tested on *Enterobacteriaceae* isolated from fresh produce

Antibiotic class	Antibiotic	Abb.	[Disc] (µg)	Supplier
Penicillin's	Ampicillin	AP 10C	10	Davies diagnostics
Chloramphenicol	Chloramphenicol	C 30C	30	Davies diagnostics
Fluoroquinolones	Ciprofloxacin	CIP 5C	5	Davies diagnostics
Cephalosporins	Cloxacillin	CX 5C	5	Davies diagnostics
Aminoglycoside	Gentamycin	GM 10C	10	Davies diagnostics
Cephamycins	Cefoxitin	FOX 30C	30	Davies diagnostics
Cephalosporins	Imipenem	IMI 10C	10	Davies diagnostics
Sulphonamides	Trimethoprim-sulfamethoxazole 1.25/23.75	TS 25C	25	Davies diagnostics
Tetracycline	Tetracycline	TE 30	30	Thermofisher

The choice of antibiotics used in this study was made based on the frequency of the antibiotic used in the environment, clinical sector or for ESBL producing *Enterobacteriaceae* conformational purposes (Adwan *et al.*, 1998; Livermore *et al.*, 2002; Sarmah *et al.*, 2006; Ehlers *et al.*, 2009; Bush and Jacoby, 2010; Kanj and Kanafani, 2011; EUCAST, 2017a). The breakpoint values are specific to the organism and antibiotic that the organism is exposed to (EUCAST, 2018). The EUCAST breakpoints values, that determine whether an organism is susceptible or resistant to an antibiotic, was used for this study. Because EUCAST is a standard that focuses on antibiotics resistance in patients infected by bacteria, some of the important environmental antibiotics are not listed in the EUCAST standard e.g. Tetracycline. Cloxacillin resistance is well known and is therefore not included in the EUCAST or CLSI standard. However, Cloxacillin is included in this study to determine whether Cloxacillin resistance in bacteria has spread to bacteria present on the South African fresh

produce as well. The breakpoint values for Tetracycline are from the Clinical and Laboratory Standards Institute (CLSI) subcommittee in antimicrobial susceptibility testing (AST) (CLSI, 2017).

Table 4.3 A summary of the breaking points used as references for the antibacterial agents used in this study (CLSI, 2017; EUCAST, 2018)

Antibiotic agent	Abbreviation	Zone diameter breakpoints (mm)	
		S >	R <
Ampicillin	AP	14	14
Chloramphenicol	C	17	17
Ciprofloxacin	CIP	26	24
Cloxacillin	CX	-	-
Gentamycin	GM	17	14
Cefoxitin	FOX	19	19
Imipenem	IMI	22	16
Trimethoprim-sulfamethoxazole 1.25/23.75	TS	14	11
Tetracycline	TE	15	11

S = susceptible, R = Resistant

4.4. RESULTS AND DISCUSSION

Screening for ESBL Enterobacteriaceae

A total of 150 fresh produce samples were tested of which 50% of the samples was lettuce. Cabbage and spinach each represented 13% and 10% were tomatoes. Green peppers and green beans were 7% of the total samples analysed. The screening for ESBL *Enterobacteriaceae* was conducted by using ESBL ChromID agar (Biomeriuex, South Africa). All samples were macerated and enriched in EE broth before streaking onto the ESBL ChromID agar. The overview of the percentage produce samples of which presumptive positive ESBL producing organisms were isolated are given in Figure 4.2, 4.3 and 4.4. The three repetitions are shown separately. Only two products were tested per site. The products that were not tested, are indicated in a bar graph below the x-axis.

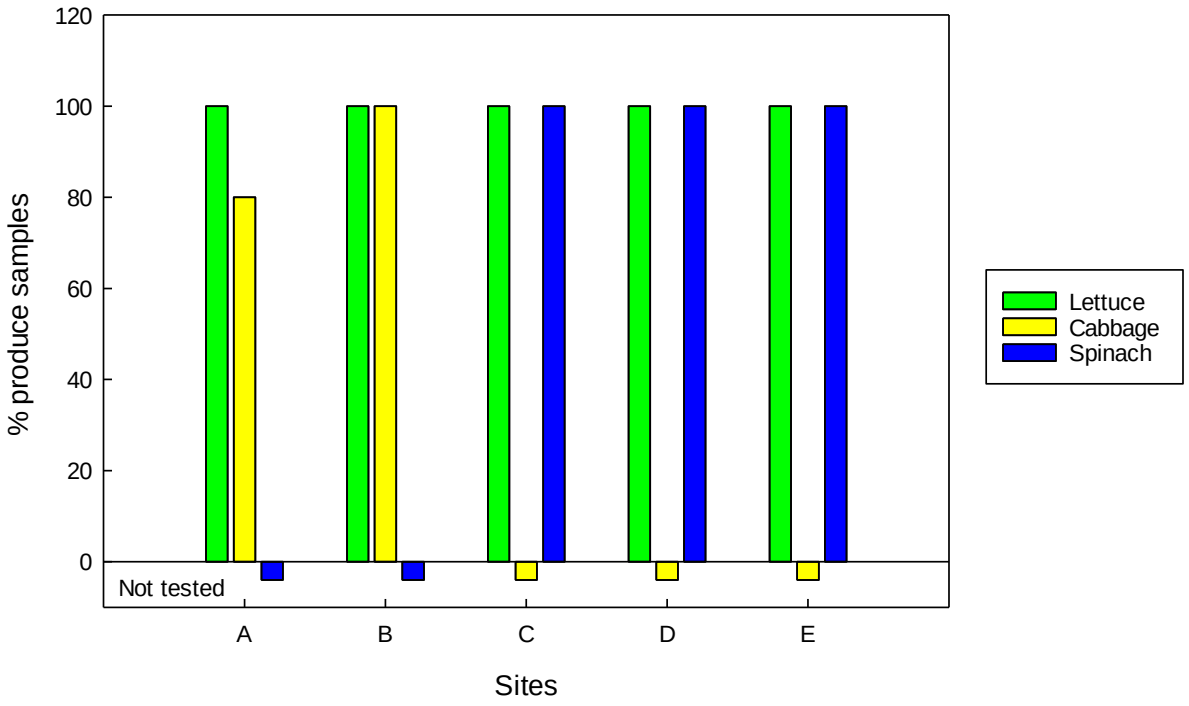

Figure 4.22 The distribution of the produce samples from which presumptive positive ESBL producing colonies were isolated during repetition 1. (The produce types not tested at a particular site are indicated in a bar under the x-axis)

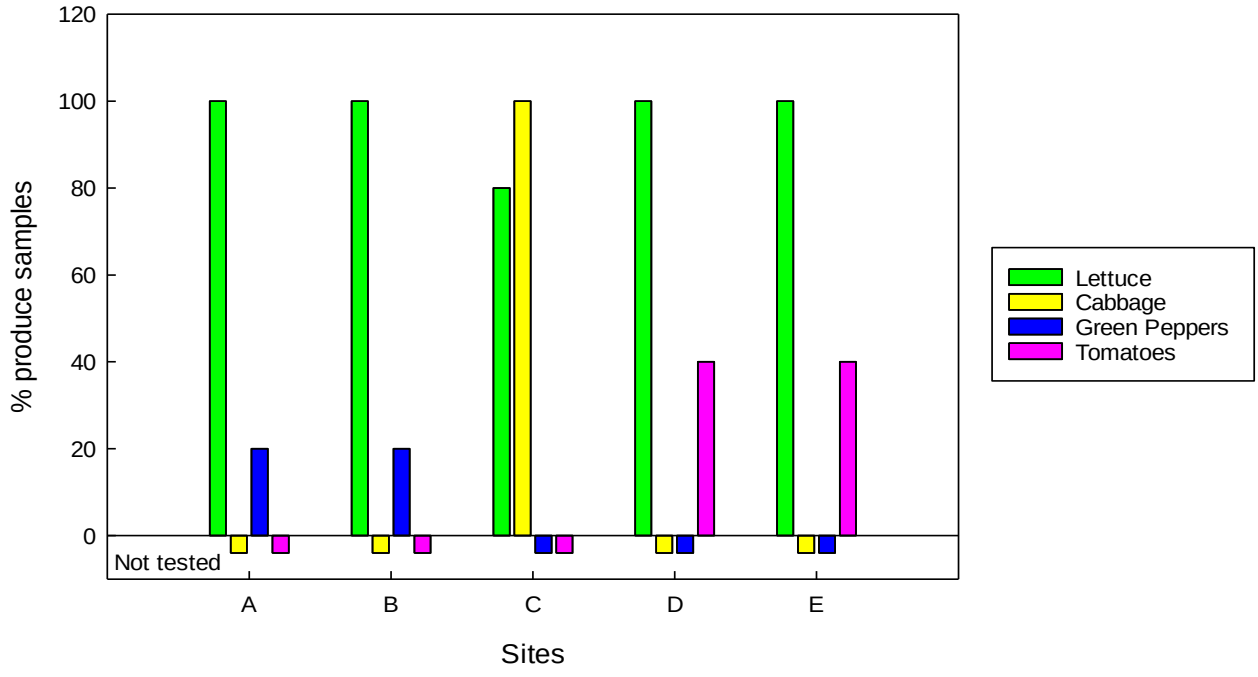

Figure 4.23 The distribution of the produce samples from which presumptive positive ESBL producing colonies were isolated during repetition 2. (The produce types not tested at a particular site are indicated in a bar under the x-axis)

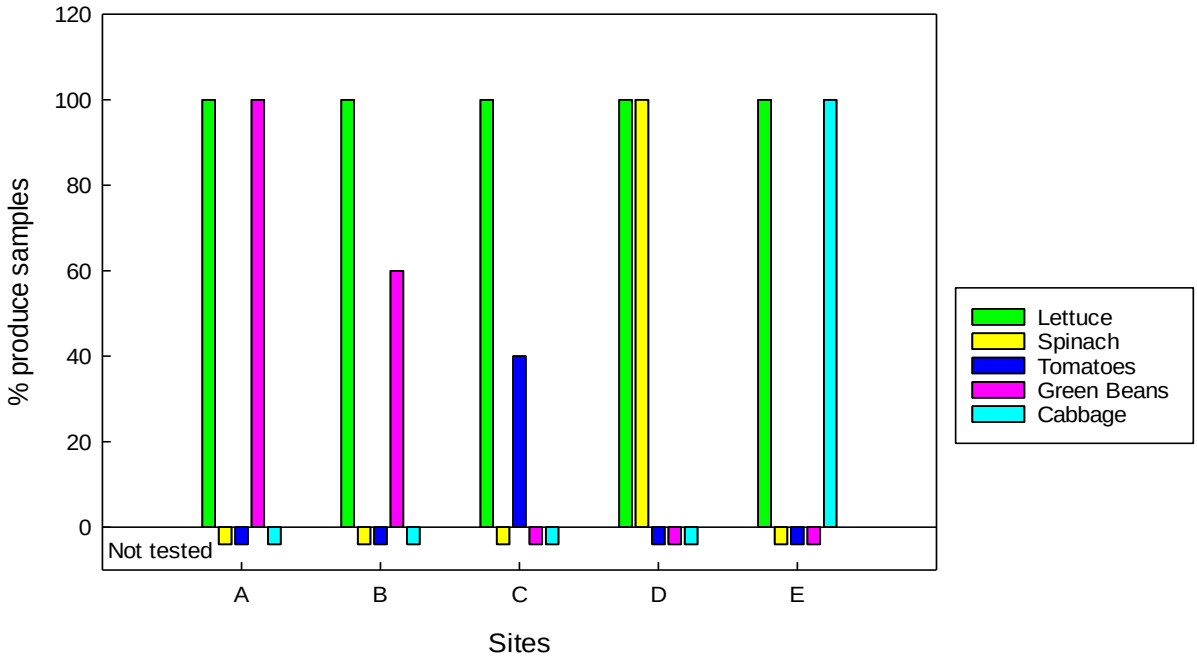

Figure 4.24 The distribution of the produce samples from which presumptive positive ESBL producing colonies were isolated during repetition 3. (The produce types not tested at a particular site are indicated in a bar under the x-axis)

An unexpected high prevalence (98%) of presumptive positive ESBL producing organisms were observed on the fresh produce during the first repetition (Figure 4.2). ESBL producers were isolated from all lettuce and spinach samples. This is true for all the repetitions (Figures 4.2, 4.3 and 4.4). The prevalence of presumptive positive ESBL producing producers in green peppers and tomatoes was lower than for the other fresh produce products. Similar observations were made for the enumeration of coliforms for tomatoes and green peppers in Chapter 3. The coliform counts on these produce types were lower in comparison with the lettuce counts. The lower pH of the tomatoes and the common use of pesticides on green peppers could have influenced the lower prevalence of ESBL producers on tomatoes and green peppers in comparison to the other products (Beuchat, 1996). A 70% presumptive positive ESBL producing organism prevalence was observed in repetition 2 (Figure 4.3). A similar tendency was also observed in repetition 3. A lower prevalence of ESBL producers was observed in the tomatoes. In repetition 3, 90% of the produce yielded presumptive positive ESBL producers. Overall, 86% of all the fresh produce that was screened for ESBL producers yielded presumptive positive colonies on the ESBL ChromID agar (Biomeriuex).

Identification of ESBL Enterobacteriaceae using MALDI-ToF

After screening for ESBL producing organisms, a total of 416 colonies were isolated from the 150 fresh produce samples. Only 38% (n = 158) of the total isolates were selected for further identification using MALDI-ToF. The summarised results of the strain identification from the MALDI-ToF are presented in Figure 4.5. Each of the genera identified is expressed as a percentage of the total isolates tested (n = 158).

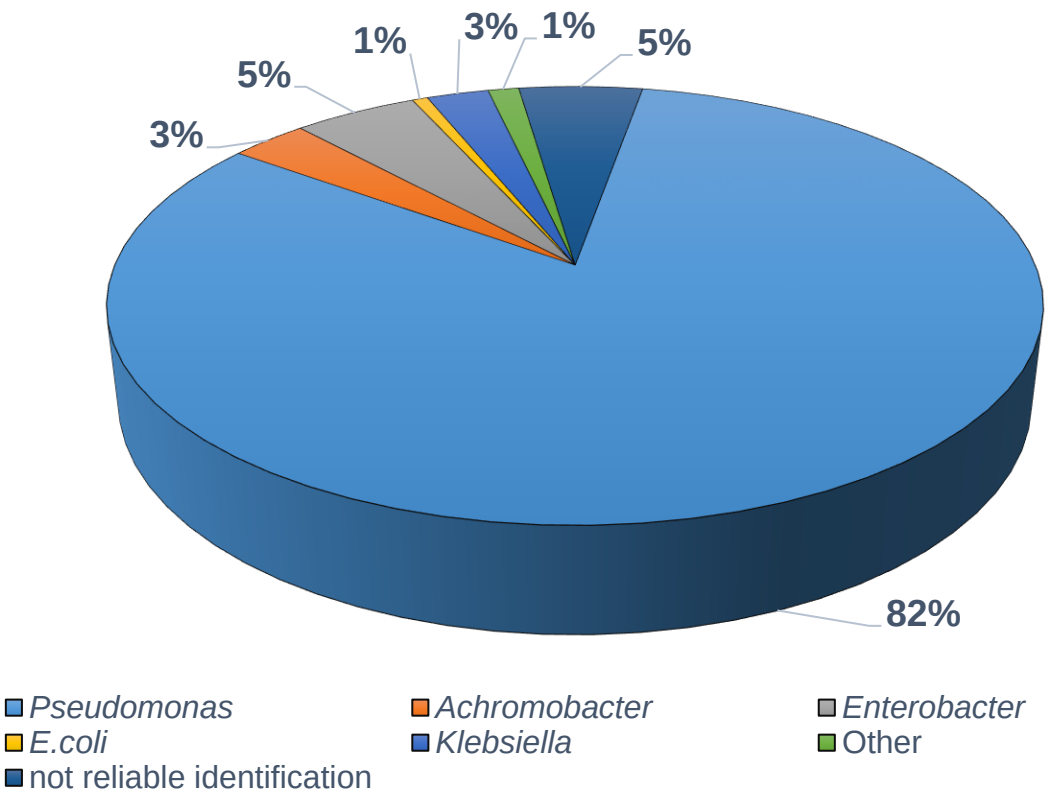

Figure 4.25 The identification of the 158 presumptive positive ESBL producing isolates that were subjected to MALDI-ToF identification expressed as a percentage of the total (n = 158).

A large percentage of non-*Enterobacteriaceae* was isolated. Only 8.2% (13/158) of the isolates identified were from the *Enterobacteriaceae* family. Eighty-six percent (136/158) of the isolates resulted in being non-*Enterobacteriaceae*. The remaining 5.7% of the isolates could not be identified. Four isolates of the *Enterobacteriaceae* were identified as *Klebsiella pneumoniae* originally isolated from cabbage and spinach. One *E. coli* was isolated from lettuce and three *Enterobacter* strains were isolated from lettuce and tomatoes. Five *Enterobacter asburiae* strains were isolated from lettuce, spinach and green beans. The non-*Enterobacteriaceae* isolates included *Achromobacter*, *Stenotrophomonas maltophilia* and *Pseudomonas* that accounted for 82% of the total of 158 isolates (Figure 4.5).

Confirmation of ESBL Enterobacteriaceae

After the presumptive positive ESBL producing organisms has been identified both *Enterobacteriaceae* and non-*Enterobacteriaceae* were subjected to phenotypical ESBL confirmation using the combination diffusion disk test (EUCAST). Figure 4.6 is an example of the typical results of the disk diffusion test yielded and how the zone diameter was measured.

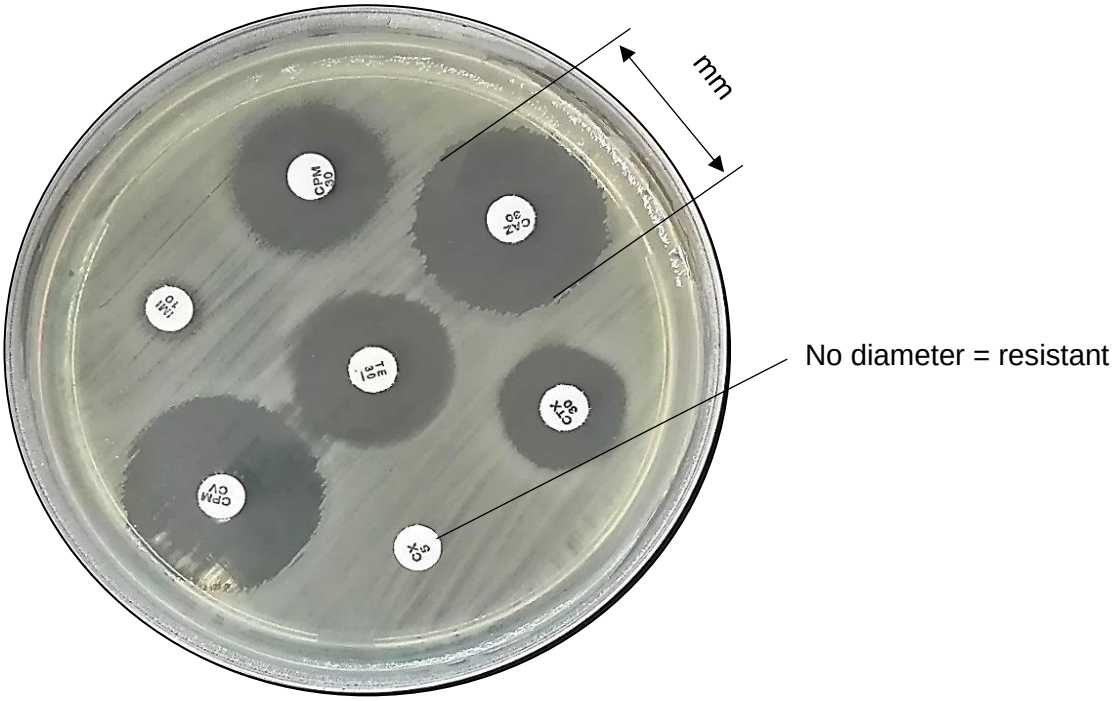

Figure 4.26 A combination disk diffusion test example with the antibiotic disks after 18 hours incubation.

The 8.2% (13/158) of the 158 isolates identified using MALDI-ToF were confirmed as *Enterobacteriaceae* and was subjected to the combination disk diffusion test (EUCAST, 2017a). The results are presented in Table 4.4. The confirmed ESBL producing *Enterobacteriaceae* were mostly isolated from lettuce and cabbage.

Table 4.4 The confirmed ESBL producing *Enterobacteriaceae* results using the combination disk diffusion test (EUCAST)

					Disk diffusion	***Molecular confirmation***		
Sample date	Site	Product	Enterobacteria-ceae	Isolate nr	ESBL confirm	*bla* SHV	bla CTX-M	*bla* TEM
06/11/2017	B	Cabbage	*Klebsiella pneumoniae*	294	+	+	+	+
06/11/2017	B	Cabbage	*Klebsiella pneumoniae*	160	+	-	+	+
06/11/2017	B	Cabbage	*Klebsiella pneumoniae*	999	+	-	+	+
05/12/2017	C	Spinach	*Klebsiella pneumoniae*	974	+	-	+	+
05/02/2018	E	Tomatoes	*Enterobacter cloacae*	542	+	-	-	-
12/03/2018	A	Lettuce	*Enterobacter cloacae*	601	+	-	-	-
16/04/2018	E	Lettuce	*Enterobacter asburiae*	558	+	+	-	+

Seven out of 13 (53.8%) *Enterobacteriaceae* strains were confirmed as ESBL producing *Enterobacteriaceae*. Four of the seven strains (57.1%) were *Klebsiella pneumonia* and were isolated from cabbage at site B and C during repetition 1 (Table 4.4). The rest of the confirmed ESBL producing *Enterobacteriaceae*, 3/7 (42.8%) were *Enterobacter cloacae* and *Enterobacter asburiae* that were isolated from tomatoes and lettuce during repetitions 2 and 3 (Table 4.4).

EUCAST (2018) does not include breakpoint values for *Pseudomonas.* This is likely because *Pseudomonas* is a low infection risk organism and therefore is not routinely tested. *Pseudomonas* classifies as part of the *Proteobacteria* family while ESBL status confirmation is only reliable for organisms that are part of the *Enterobacteriaceae* family. However, non-*Enterobacteriaceae* isolates that showed similar behaviour to *Enterobacteriaceae* in terms the growth in presence of Ceftazidime, Cefotaxime and Cefepime in combination with clavulanic acid, are presented in Table 4.5.

Table 4.5 The non-*Enterobacteriaceae* results that was obtained using the combination disk diffusion test for the confirmation of ESBL producing bacteria

					Disk diffusion	**Molecular confirmation**		
Sample date	Site	Product	Organism	Isolate nr	ESBL similar	*bla* SHV	bla CTX-M	*bla* TEM
06/11/2017	A	Cabbage	*Pseudomonas otitidis*	758	+	-	-	-
06/11/2017	A	Lettuce	*Achromobacter insolitus*	210	+	+	+	+
05/12/2017	C	Spinach	*Pseudomonas putida*	568	+	-	+	+
15/01/2018	B	Lettuce	*Pseudomonas chlororaphis*	473	+	-	-	+
13/02/2018	C	Lettuce	*Pseudomonas putida*	366	+	-	+	+
13/02/2018	C	Lettuce	*Pseudomonas putida*	896	+	-	+	+
13/02/2018	C	Lettuce	*Achromobacter spanius*	302	+	-	-	-
29/01/2018	D	Tomatoes	*Pseudomonas koreensis*	308	+	-	-	-
12/03/2018	A	Lettuce	*Pseudomonas putida*	849	+	-	-	+
12/03/2018	A	Green beans	*Stenotrophomonas maltophilia*	137	+	-	-	-
12/03/2018	A	Green beans	*Stenotrophomonas maltophilia*	730	+	-	-	-
19/03/2018	B	Lettuce	*Pseudomonas putida*	458	+	n.a.	n.a.	n.a.
16/04/2018	E	Lettuce	*Achromobacter xylosoxidans*	839	+	-	-	-
16/04/2018	E	Lettuce	*Pseudomonas putida*	409	+	+	+	+
16/04/2018	E	Lettuce	*Pseudomonas putida*	898	+	+	+	+
16/04/2018	E	Cabbage	*Pseudomonas putida*	478	+	+	-	+
16/04/2018	E	Cabbage	*Pseudomonas putida*	788	+	+	-	+
16/04/2018	E	Cabbage	*Pseudomonas monteilii*	057	+	+	-	+
16/04/2018	E	Cabbage	*Pseudomonas mendocina*	887	+	+	+	+

The organisms isolated from the fresh produce that were confirmed as ESBL producers with the antibiotic susceptibility combination disk diffusion test were also subjected to molecular confirmation using PCR. The ESBL genes of interest (bla_{SHV}, $bla_{CTX\text{-}M,}$ *and* bla_{TEM}) were amplified in the positive control isolate (Figure 4.7). Preliminary work was completed using a 1.2% agarose gel to eliminate

isolates that were negative for all three ESBL genes of interest. Isolates that were positive or suspected to be positive for one or more of the ESBL genes were visualised using the LabChip GX II Touch microfluidic electrophoresis instrument (PerkinElmer, South Africa). A summary of the molecular results that were visualised by the Labchip instrument are included in Tables 4.4 and 4.5.

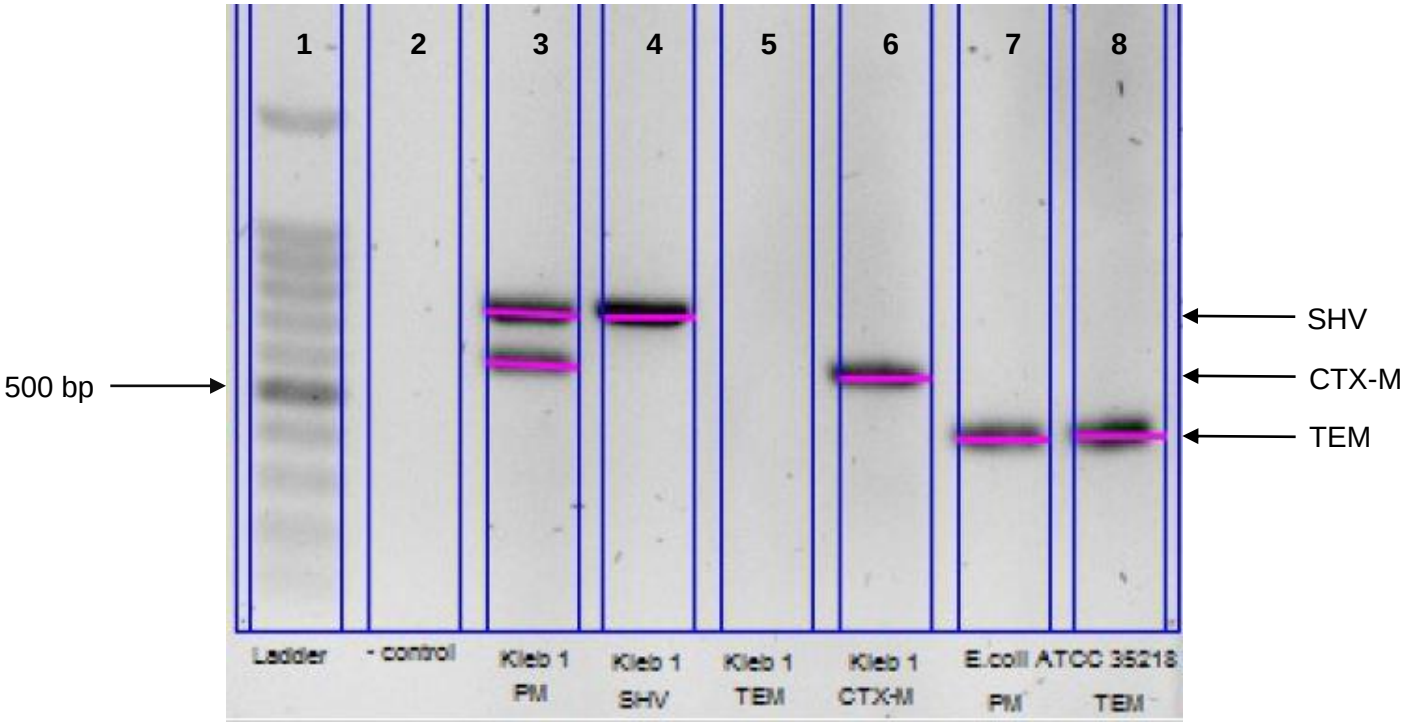

Figure 4.27 A 1.2% agarose gel detecting the *bla*$_{SHV}$, *bla*$_{CTX-M,}$ *and bla*$_{TEM}$ confirming the presence of the ESBL genes in the positive control. Lane 1 = 100 bp ladder, Lane 2 = negative control, Lane 3 = *K. pneumonia* PM, Lane 4 = *K. pneumonia bla*$_{SHV}$ amplified, Lane 5 = *K. pneumonia bla*$_{TEM}$ amplified, Lane 6 = *K. pneumonia bla*$_{CTX-M}$ amplified, Lane 7 = *E. coli* ATCC 35218 PM, Lane 8 = *E. coli* ATCC 35218 *bla*$_{TEM}$ amplified. *PM = primer mix used for amplification.

Figure 4.7 is a visual representation of the 1.2% agarose gel with the amplification products of the positive controls. *K. pneumonia* contained *bla*$_{SHV}$, *bla*$_{CTX-M}$ (lanes 4 and 6), but not *bla*$_{TEM}$ (lane 3). *E. coli* ATCC 35218 contained *bla*$_{TEM}$ (lane 8) but not *bla*$_{SHV}$ or *bla*$_{CTX-M,}$ (lane 7). Therefore a 50:50 mixture was prepared to use as a positive control for further tests.

Antibiotic resistance profiles

Table 4.6 includes the antibiotic resistance profiles of all the *Enterobacteriaceae* that were confirmed as ESBL producers and non-ESBL producing *Enterobacteriaceae* (n = 13). The isolates were subjected to antibiotics from eight different classes including Penicillin, Chloramphenicol,

Fluoroquinolones, Cephalosporins, Aminoglycoside, Cephamycins, Sulphonamides and Tetracycline. The results were interpreted using the breaking point tables summarised in Table 4.3.

Table 4.6 The *Enterobacteriaceae* results for the antibiotic susceptibility testing (EUCAST)

Site	Product	*Enterobacteria-ceae*	nr	Ampicillin	Cefoxitin	Chloramphenicol	Cloxacillin	Ciprofloxacin	Gentamycin	Imipenem	Tetracycline	TS
B	Cabbage	*K. pneumoniae*	294	R	R	R	R	R	R	S	R	R
B	Cabbage	*K. pneumoniae*	160	R	S	S	R	R	R	S	R	R
B	Cabbage	*K. pneumoniae*	999	R	S	S	R	R	R	S	R	R
C	Spinach	*K. pneumoniae*	974	R	S	S	R	R	S	S	R	R
B	Lettuce	*E. coli*	105	R	S	R	R	S	S	S	S	S
E	Lettuce	*E. cloacae*	012	S	R	S	R	S	S	S	S	S
E	Tomatoes	*E. cloacae*	542	R	R	S	R	S	S	S	S	S
A	Lettuce	*E. cloacae*	601	R	R	R	R	S	S	S	S	R
B	Lettuce	*E. asburiae*	155	R	R	R	R	S	S	R	S	S
B	Green beans	*E. asburiae*	853	R	R	R	R	R	R	R	S	R
D	Lettuce	*E. asburiae*	625	R	R	R	R	R	S	R	S	R
D	Spinach	*E. asburiae*	396	R	R	R	R	R	S	R	S	R
E	Lettuce	*E. asburiae*	558	R	R	R	R	R	S	S	R	R

R = Resistant, S = Susceptible, TS = Trimethoprim-sulfamethoxazole 1.25/23.75

Twelve of the thirteen *Enterobacteriaceae* isolated from fresh produce were also resistant to three or more antibiotics in different classes. Therefore, 92% of the *Enterobacteriaceae* isolated can be considered multidrug resistant (Table 4.6). Seven (53.8%) of these isolates are also confirmed ESBL producing *Enterobacteriaceae* (Table 4.4). There are many factors contributing to antibiotic resistance in the environment. Antibiotic misuse in the clinical sector is not the only factor (Cantón & Coque, 2006). Antibiotics are used in the agricultural environment as growth promoters in feed, for feed efficiency and for animal disease treatment, especially Tetracycline (Sarmah *et al.*, 2006). The antibiotic agents are fed to the animal either via feed, water, by injection, paste or orally. However, the antibiotics that are applied orally have the greatest effect on antibiotic resistance (Sarmah *et al.*,

2006). A few studies have shown that 30-90%, depending on the antibiotic, is not absorbed by the gut of the animal when administered and is being excreted into the environment (Elmund *et al.*, 1971; Alcock *et al.*, 1999; Sarmah *et al.*, 2006). In European countries, the use of antibiotics as growth promoters was banned in the early 1970's because of this effect (Heuer *et al.*, 2011).

During the 1970's, the *bla*$_{SHV}$ and *bla*$_{TEM}$ genes were considered the genes of concern. After a widespread pandemic of CTX-M producing organisms in the 2000's, it was considered the new gene of concern that causes the resistance of ESBL producing *Enterobacteriaceae* particularly in the organism *E. coli* and *K. pneumonia* (Cantón & Coque, 2006; Falagas and Karageorgopoulos, 2009). Antibiotics that were used for the treatment of ESBL related infections in the past, but recently showed resistance against some isolates includes Cephamycins, Fluoroquinolones, Aminoglycosides, Tetracyclines and a combination of Trimethoprim/sulfamethoxazole. This is confirmed in this study. *Klebsiella pneumonia* isolated from cabbage (isolates 160 and 999) were resistant against Gentamycin (Aminoglycoside), Tetracycline (Tetracycline), Trimethoprim-sulfamethoxazole (Sulphamides) and Ciprofloxacin (Fluoroquinolone) whereas isolate 294 was resistant to Cefoxitin (Cephamycin) as well (Table 4.6).

In 2006 a novel *Pseudomonas* species, which was named *Pseudomonas otitidis*, was isolated from clinical samples from patients with infected ears that suffered from acute otitis externa (Clark *et al.*, 2006). Six *Pseudomonas otitidis* were isolated from lettuce and cabbage during this study. Clark *et al.* (2006) reported that the *Pseudomonas otitidis* isolated from the patients was resistant against Tetracycline and Chloramphenicol but susceptible to Gentamycin (Aminoglycoside) and Ciprofloxacin (Fluoroquinolones). Similar results were observed during this study. *Pseudomonas otitidis* (isolate 281, 758, 151, 728, 951 and 858) is most likely treatable with Gentamycin (Aminoglycoside), Ciprofloxacin (Fluoroquinolone) and Tetracycline (Tetracycline), but not Chloramphenicol (Chloramphenicol).

4.5. CONCLUSION

The aim of this study was to screen for the presence of ESBL producing *Enterobacteriaceae* and their prevalence in fresh produce sold at informal retailers in the Cape Town Metropolitan area, South Africa. Looking at the initial percentage of presumptive positive ESBL producers it became worrisome to see such a high prevalence (86% of a total number of tested samples) of presumptive ESBL producers. These organisms were present on lettuce, cabbage, spinach, tomatoes, green beans and green peppers that are consumed raw. However, after the MALDI-ToF identification of the isolated presumptive positive ESBL producers, it became clear that only 8% of the isolates were indeed *Enterobacteriaceae*. These isolates were confirmed as ESBL producers via phenotypical confirmation. After the EUCAST combination disk diffusion test, only half of the isolates were confirmed as ESBL producing *Enterobacteriaceae*. The confirmed ESBL producing *Enterobacteriaceae* consisted of four *Klebsiella pneumonia* strains isolated from lettuce and spinach,

one *Enterobacter cloacae* isolated from tomatoes and one *Enterobacter asburiae* isolated from lettuce. *Klebsiella pneumonia* is not a routinely tested pathogen in food but has high priority in the clinical sector for infection prevention and treatment because it is a pathogen known for carrying multidrug-resistant properties. This makes treatment options extremely limited. This was confirmed in this study where the most resistant isolated strain was resistant against Gentamycin, Cefoxitin, Cloxacillin, Chloramphenicol, Ciprofloxacin, Ampicillin, Trimethoprim-sulfamethoxazole and Tetracycline. To see a prevalence rate of 3% of all the (158) samples being tested being *Klebsiella pneumoniae* which was confirmed to be ESBL producing and being resistant to seven different classes of antibiotics, is concerning. Direct contamination from food handlers or faecal contamination in washing water or irrigational water could have been the cause of the contamination with multi-drug resistant, ESBL producing *Klebsiella pneumonia,* also known as the “super-bug” according to the CDC (CDC, 2013).

The environmental organisms such as *Pseudomonas* that also contained ESBL genes (*bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{TEM}$) are organisms that are often overlooked for the cause of further dissemination of resistant genes into the environment. *Pseudomonas, Enterobacter cloacae, Citrobacter freundii* and *Serratia marcescens* are the organisms whose resistance were initially observed (Philippon *et al.*, 2002). These are possibly the organisms that are historically responsible for the rapid spread of ESBL producing organisms (Cantón & Coque, 2006). The concern is that these environmental organisms could share their resistance genes with foodborne pathogens in the same environment (human gut, soil or water), which could then also use the resistance genes for their own survival and benefit.

A recommendation for further studies would be to isolate plasmid DNA rather than genomic DNA from the isolates to confirm whether the resistant genes are carried on plasmids or not. This can assist in the risk analysis of environmental bacteria carrying antibiotic resistant genes on their plasmid which would increase the chance of the horizontal gene transfer of antibiotic resistant genes to foodborne pathogens.

This study provided a snapshot of the current situation regarding the presence and prevalence of ESBL producing *Enterobacteriaceae* in fresh produce in the informal market in the Cape Town metropolitan area, South Africa. This study confirmed the presence of ESBL producing *Enterobacteriaceae* in fresh produce. However, the prevalence thereof is relatively low (4%). The study also confirmed that the prevalence of multidrug resistant organisms in the ESBL producing *Enterobacteriaceae* was extremely high (100%, 7/7). The presence of ESBL producing *Enterobacteriaceae* and the possibility of the further dissemination of ESBL genes into the environment, thereby increasing the current antibiotic resistance problem, cannot be ignored.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

The consumption of fresh produce is increasing globally. With the increase in consumption, the number of foodborne related outbreaks linked to fresh produce is also increasing (Leon *et al.*, 2009). The Food and Agricultural Organization and the World Health Organization classified leafy green vegetables including lettuce, spinach, cabbage and fresh herbs as level 1 priority based on their concern for microbiological contamination. Tomatoes were prioritised as level 2 (FAO & WHO, 2008). Fresh produce is consumed raw, which increases the risk to consumers' health when it is contaminated. It is therefore important to know the current microbiological safety status of fresh produce in the Western Cape, South Africa, where very limited information is available on the informal market. The possibility of antibiotic resistant organisms present in fresh produce also increases the health risk of the consumer, however, very limited information is available on the prevalence of antibiotic resistant organisms present on fresh produce in South Africa.

The aim of the study was to determine the microbiological safety of selected fresh produce (lettuce, spinach, cabbage, tomatoes, green peppers and green beans) sold in the informal market at selected points in the Cape Town Metropolitan area in South Africa. This was achieved by evaluating the overall microbiological quality of the selected fresh produce along with the detection of high-risk pathogens frequently associated with fresh produce. The presence and prevalence of an emerging opportunistic pathogen, Extended-Spectrum β-Lactamase (ESBL) producing *Enterobacteriaceae*, was also evaluated that add on to the microbiological risk of fresh produce.

The objective of the first part of the study was to enumerate hygiene indicator microorganisms such as coliforms, *Enterobacteriaceae* and *E. coli.* The food safety of the fresh produce was evaluated by detecting specific foodborne pathogens (Shiga toxin-producing *E. coli (STEC)*, *Salmonella* spp. and *Listeria monocytogenes)* in selected fresh produce sold at informal retailers in the Cape Town Metropolitan area. Five informal street vendors were identified and sampled each week. The coliform and *Enterobacteriaceae* counts for all the products at all sites were well over the advised microbiological limits (DoH, 2000). Green peppers and tomatoes, however, were the exception and were within the advised limit. No *Salmonella* or *Listeria monocytogenes* were detected in any of the fresh produce, however, one lettuce sample tested positive for STEC. Out of a total of 150 products tested, 11.33% of fresh produce tested contained *E. coli* at average levels of $2.7x10^3$ cfu.g^{-1}. The presence of *E. coli* occurred sporadically suggesting that *E. coli* contamination could be linked to the poor post-harvest handling.

The objective of the second part of the study was to determine the antibiotic resistant profiles of Extended-Spectrum β-Lactamase-producing *Enterobacteriaceae* isolated from selected fresh produce. This study provided a snapshot of the current situation regarding the presence and prevalence of ESBL producing *Enterobacteriaceae* in fresh produce in the informal market in the

Cape Town metropolitan area, South Africa. The presence of ESBL producing *Enterobacteriaceae* was confirmed. However, the prevalence thereof was relatively low (4% of total samples). The antibiotic resistance of ESBL producing *Enterobacteriaceae* was determined. The results indicated that multi-drug resistance was very common amongst the ESBL producing *Enterobacteriaceae* with prevalence of 100% (6/6 isolates). Multi-drug resistance is confirmed in an isolate if the organism is resistant to three or more antibiotics in different cases which makes treating the infection caused by the organism extremely limited (Zurfluh *et al.*, 2015). The presence of ESBL genes in non-*Enterobacteriaceae/*environmental organisms should not be overlooked. Environmental organisms containing ESBL related genes (such as $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}) have the potential to disseminate resistant genes into the environment (Blaak *et al.*, 2014). This creates an opportunity for resistant genes to be utilised by opportunistic pathogens. It is important to acknowledge the fact that ESBL producing *Enterobacteriaceae* can be present on fresh produce and that these organisms might have the ability to transfer their resistance genes to opportunistic foodborne pathogens, if present.

It is recommended for further studies to isolate the plasmid DNA of the ESBL producing organisms to determine whether the resistant genes are carried on the plasmid or not. This can clarify the risk possed by environmental bacteria carrying antibiotic resistant genes. If the genes are present on the plasmid DNA the possibility of dissemination of antibiotic resistant genes to foodborne pathogens might be greater than if the genes are on the genomic DNA.

Although the general microbiological counts were high and above the limits advised by the South African Department of Health, there were no pathogens present, except for one lettuce sample that tested positive for Shiga toxin-producing *E. coli*. The presence of Extended-Spectrum β-Lactamase producing *Enterobacteriaceae* should be acknowledged although the prevalence is still relatively low (4% of a total number of tested samples). All confirmed ESBL producing *Enterobacteriaceae* were however resistant to three or more antibiotics in different antibiotic classes. Not only have these organisms gained the ability to counteract the activity of the most widely used antibiotics (third generation cephalosporins) but are also resistant to other classes of antibiotics making the treatment of infections caused by these organisms extremely limited.

It is clear throughout the study that the sporadic distribution of *E. coli* and inconsistent microbiological counts of the fresh produce is an indication of inconsistencies of the quality and safety of fresh produce in the informal market. There is limited/no information about the transportation, origin or handling of the fresh produce which increases the difficulty of regulating the safety of the fresh produce that is being sold. Regardless of the high hygiene indicator counts and the presence of *E. coli* in sporadically occurring events, there were no pathogens detected (excluding one event). Therefore, there is no evidence that the fresh produce tested in this study is unsafe for consumption. However, it is clear that there is a need for more surveillance studies in the informal sector to evaluate the food safety risk of fresh produce, a high-risk food group, especially in South Africa.

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