Prevalence and characterisation of antimicrobial resistant Enterobacteriaceae in fresh vegetables from farm to retail in the Gauteng Province of South Africa

by

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Declaration

I, the undersigned, declare that the thesis, which I hereby submit for the degree Philosophiae Doctor in Biotechnology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Loandi Richter

This thesis is dedicated to my parents

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Jeremiah 29:11: "For I know the plans I have for you," declares the Lord, "plans to prosper you and not to harm you, plans to give you hope and a future."

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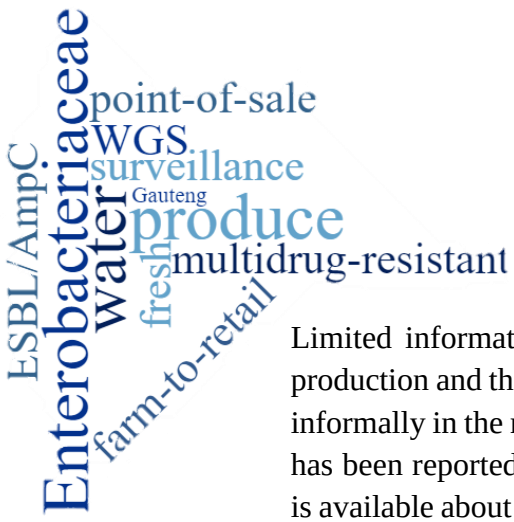
Thesis Summary



A potential food safety risk is evident through persistence and survival of human pathogens on fresh produce for extended periods of time. Additionally, contaminated irrigation water has been reported as a major source of contamination in fresh produce production.

Limited information is available regarding irrigation water used during crop production and the microbiological safety of the fresh produce sold formally and informally in the markets of South Africa. Furthermore, antimicrobial resistance has been reported as an emerging human health threat, yet limited information is available about microbial dissemination within the water-plant-food interface. No studies have reported on the prevalence of Enterobacteriaceae, with expanded antimicrobial resistance in fresh produce supply chains within South Africa.

The main aim of this thesis was to evaluate the microbiological safety status and prevalence of multidrug resistant potential pathogens in South African fresh produce supply chains, focusing on the densely populated Gauteng Province.

The thesis consists of eight chapters (Figure 1), of which Chapter 2 presents a critical review of the existing body of literature describing the significance of Enterobacterales within fresh produce supply systems and fresh produce microbiological safety. The review further highlights antimicrobial resistance from a food safety perspective. Chapters 3, 4, 5, 6, and 7 focuses on the three hypothesis statements addressed in the overall project. Based on the results from the overall project, the thesis is presented as a compilation of publications, as Chapters 3, 4, 5, 6 and 7 have been published.

- **Chapter 3:**
  Richter, L., du Plessis, E.M., Duvenage, S., and Korsten, L**. (2021).** High prevalence of multidrug resistant *Escherichia coli* isolated from fresh vegetables sold by selected formal and informal traders in the most densely populated Province of South Africa. *J. Food Sci.* 86, 161–168. doi:10.1111/1750-3841.15534.

- **Chapter 4:**
  Richter, L., du Plessis, E.M., Duvenage, S., and Korsten, L. **(2019).** Occurrence, identification, and antimicrobial resistance profiles of extended-spectrum and AmpC β-lactamase-producing Enterobacteriaceae from fresh vegetables retailed in Gauteng Province, South Africa. *Foodborne Pathog. Dis.* 16, 421–427. doi:10.1089/fpd.2018.2558.

- **Chapter 5:**
  Richter, L., du Plessis, E.M., Duvenage, S., and Korsten, L. **(2021).** Microbiological safety of spinach throughout commercial supply chains in Gauteng Province, South Africa and characterisation of isolated multidrug resistant *Escherichia coli*. Journal of Applied Microbiology. doi: 10.1111/jam.15357.

- **Chapter 6:**
  Richter, L., du Plessis, E. M., Duvenage, S., and Korsten, L. **(2020).** Occurrence, phenotypic and molecular characterization of extended-spectrum- and AmpC- β-lactamase producing Enterobacteriaceae isolated from selected commercial spinach supply chains in South Africa. *Front. Microbiol.* 11, 1–10. doi:10.3389/fmicb.2020.00638.

- **Chapter 7:**
  Richter, L., du Plessis, E. M., Duvenage, S., Allam, M., Ismail, A., and Korsten, L. **(2021).** Whole Genome Sequencing of Extended-Spectrum- and AmpC- β-Lactamase-Positive Enterobacterales Isolated From Spinach Production in Gauteng Province, South Africa. Front. Microbiol. 12. doi:10.3389/fmicb.2021.734649.

Abstract

Contaminated fresh produce has increasingly been implicated in foodborne disease outbreaks with antimicrobial resistance reported as a major emerging health threat. This study aimed to determine the microbiological quality and prevalence of potential pathogenic *Escherichia coli* and foodborne pathogens (*Salmonella* spp., and *Listeria monocytogenes*) in fresh produce retailed formally and informally, as well as two commercial spinach production systems on farm, through processing and up to retail, in Gauteng, the most densely populated province of South Africa (SA). Additionally, the prevalence and molecular characteristics of multidrug resistant extended-spectrum β-lactamase (ESBL) and AmpC-producing Enterobacteriaceae were investigated.

A total of 833 samples were analysed. This included 545 spinach, tomatoes, lettuce, cucumber and green beans samples purchased from formal and informal retailers in Gauteng Province. Furthermore, 288 samples were collected from two commercial spinach production scenarios with different irrigation water (river and borehole) sources. From the supply chains, spinach samples were taken at harvest, during processing and from the associated retailers. Irrigation water from each respective farm were taken at the source, storage dams, irrigation pivot point in the field and water used during processing. Lastly, soil at harvest and swab samples from contact surfaces including crates, floors and cutting surfaces throughout the respective production systems were analysed.

Coliforms, *E. coli* and other Enterobacteriaceae enumerated from fresh produce at the point of sale were mostly not significantly different between formal and informal markets, with exceptions noted on occasion. In the spinach production systems, where river water was directly used as overhead irrigation, *E. coli* was enumerated from spinach at harvest, during processing as well as from the ready-to-eat retail samples. Following selective enrichment and plating onto chromogenic media, potential pathogens were identified using matrix-assisted

laser desorption ionization time-of-flight (MALDI-TOF) analysis. In total, 17,5% (n=146) of the samples harboured *E. coli*, which included 81 samples from the point-of-sale and 65 samples from the spinach production systems. Except for one *stx2* positive *E. coli* isolate from river irrigation water, no virulence genes (*lt*, *st*, *bfpA*, *eagg*, *eaeA*, *stx1*, *stx2*, *ipaH*) were detected in any of the *E. coli* isolates (n=147) following PCR and sequencing. S*almonella* spp. isolates (n=11) were only recovered from river water samples, whilst no *Listeria* spp. were isolated from any of the samples. Source tracking showed a connection between *E. coli* in source water and on the irrigated crop using ERIC-PCR analysis within each supply chain.

Phenotypic antimicrobial resistance profiles (Kirby-Bauer disk diffusion) revealed multidrug resistance (MDR) in 38,8 % of the generic *E. coli* isolates (n=147). Overall, 16,4 % (137/833) of the samples were found to be contaminated with ESBL/AmpC-producing Enterobacteriaceae which included 95/545 vegetable samples at the point of sale and 42/288 samples throughout spinach production. Dominant species included *E. coli*, *Enterobacter cloacae*, *Enterobacter asburiae* and *K. pneumoniae* from vegetables at the point of sale and *Serratia fonticola*, *E. coli* and *K. pneumoniae* from the spinach supply chains. In total, 96.8 % (121/125) of the ESBL/AmpC-producing Enterobacteriaceae isolates were multidrug resistant. With PCR analysis, domination of the CTX-M group 9 ESBL type in isolates from vegetables at the point of sale were seen, while the CTX-M group 1 ESBL type were the most prevalent in Enterobacteriaceae from the spinach supply chains. Selected ESBL/AmpC-producing isolates (n=19) that represented critical priority pathogens listed by the World Health Organisation (WHO) isolated from the spinach supply chains were subjected to whole genome sequencing. In one *E. coli* and five *K. pneumoniae* strains, integron In191 were present. Relevant similarites to human pathogens were predicted with PathogenFinder for all 19 strains, with a confidence of 0.635- 0.721 in *S. fonticola*, 0.852 – 0.931 in *E. coli*, 0.796 – 0.899 in *K. pneumoniae* and 0.939 in the *S. enterica* strain. The presence of MDR ESBL/AmpC-producing

E. coli, *K. pneumoniae*, *S. fonticola* and *S. enterica* with confirmed similarities to human pathogens reflect the agricultural production environment link in the emergence and spread of antibiotic resistance genes.

The necessity of using clean and safe irrigation water in fresh produce production and the need for standardised microbiological safety parameters for irrigation water and ready-to-eat fresh vegetables was highlighted. For the first time, the presence of multidrug resistant ESBL/AmpC-producing Enterobacteriaceae in formally and informally retailed raw vegetables in Gauteng Province were reported. These results contribute to the global knowledge base regarding the prevalence and characteristics of ESBL/AmpC-producing Enterobacteriaceae in fresh vegetables and the agricultural environment. This will contribute towards data required for future risk analysis, and emphasises the need for mitigation strategies for combatting the spread of multidrug resistant environmental strains.

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List of Abbreviations

ALOA	Agar *Listeria* Ottavani and Agosti
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
ATCC	ATCC American Type Culture Collection
BHI	Brain heart infusion
BLEB	Buffered Listeria enrichment broth
BPW	Buffered peptone water
CCME	Canadian council of ministers of the environment
CDC	Center for Disease Control and Prevention
CFS	Centre for Food Safety
CFU	Colony Forming Units
CPI	Consumer Price Index
DAEC	Diffusely adherent *Escherichia coli*
DAFF	Department of Agriculture, Forestry and Fisheries
DDST	Double-disk synergy test
DoH	South African Department of Health
DWAF	Department of Water Affairs and Forestry
EAEC	Enteroaggregative *Escherichia coli*
EC	European Commission
EE	Enterobacteriaceae enrichment
EFSA	European Food Safety Authority
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Entero-invasive *Escherichia coli*
EMB	Eosin methylene blue
EPEC	Enteropathogenic *Escherichia coli*
ERIC-PCR	Enterobacterial Repetitive Intergenic Consensus PCR
ESBL	Extended-spectrum β-lactamase
ETEC	Enterotoxigenic *Escherichia coli*
FAO	Food and Agriculture Organization of the United Nations
FPEP	Fresh Produce Exporters' Forum
FPMs	Fresh Produce Markets
FSAI	Food Safety Authority of Ireland
GAP	Good agricultural practices
HACCP	Hazard analysis and critical control points
ICE	Integrated Conjugative element
KPC	*Klebsiella pneumoniae* carbapenemase
LSD	Least significant difference
LoD	Limit of Detection
MALDI-TOF	Matrix assisted laser desorption ionization time of flight mass spectrometry
MBL	Metallo-beta-lactamases

MDR	Multidrug resistant
MGE	Mobile genetic elements
MPN	Most probable number
NCBI	National Center for Biotechnology Information
NFPM	National Fresh Produce Markets
NICD	National Institute of Communicable Diseases
NORS	National Outbreak Reporting System
NRF	National Research Foundation
PACSA	Pietermaritzburg Agency for Community Social Action
PCR	Polymerase chain reaction
RTE	Ready-To-Eat
SADC	South African Development Community
SE	Standard error
STEC	Shiga toxin producing *Escherichia coli*
TSB	Tryptone soy broth
U.S.	United States
VRBG	Violet Red Bile Glucose
VTEC	Verotoxigenic *Escherichia coli*
WGS	Whole Genome Sequencing
WHO	World Health Organisation
WRC	Water Research Commission
WWTPs	Waste Water Treatment Plants
XLD	Xylose lysine deoxycholate

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Chapter 1

"Study the science of art. Study the art of science. Develop your senses- especially learn how to see. Realize that everything connects to everything else." -*Leonardo da Vinci*

General Introduction

Fresh produce is globally promoted as an essential component of a healthy diet, with the positive association between adequate consumption of fresh fruit and vegetables and human health being well documented (Olaimat and Holley, 2012; Claasen et al., 2016). Furthermore, amidst the COVID-19 pandemic, the value of eating healthy food has globally been promoted as vital in maintaining a healthy immune defence system. Similar to dietary guidelines globally, the South African Department of Health encourages a daily diet rich in fruit and vegetables (Vorster et al., 2013). However, due to economic constraints and a lack of awareness of its health benefits, low intake of fruit and vegetables are observed in certain communities, especially in food insecure homes of South Africa (SA) (Ronquest-Ross et al., 2015; Okop et al., 2019).

In SA, a wide range of fruit and vegetables are produced locally, with fruit accounting for up to 35% of agricultural exports [Fresh Produce Exporters' Forum (FPEF), 2021]. Fresh vegetables, on the other hand, are mainly produced and retailed nationally, although some products are exported to the South African Development Community (SADC) countries, Swaziland, the United Kingdom, the Netherlands, the Middle East and Asian markets [Department of Agriculture, Forestry and Fisheries (DAFF), 2012a; 2012b; 2016; FPEF, 2021]. Commercial producers have to comply with different food safety standards to access international markets and due to voluntary retailer requirements. In SA there is a dual food system, a well-regulated formal and a less regulated informal supply chain. The commercial farmers provide fresh produce to the formal retailers, while small-scale farmers mainly supply to the informal markets. However, some commercial produce is also retailed in the informal market as it is sold on the regional fresh produce markets. These markets cater for different

income groups/living standards measures groups [South African Audience Research Foundation (SAARF), 2012; Skinner and Haysom, 2016]. Therefore, producing, handling and retailing fresh produce often happen under different situations from being highly regulated to unregulated, making the food safety status unpredictable (Methvin et al., 2015).

Diverse bacterial communities are found on vegetables, including *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria*, with compositions that differ significantly between vegetable types (Berg et al., 2014). As an example, Leff and Fierer (2013) reported that produce types including spinach, lettuce, tomatoes, sprouts, and peppers all had high relative loads of taxa belonging to the Enterobacteriaceae family, and tended to share more similar bacterial communities, when compared to other vegetable types. As Enterobacteriaceae forms part of the normal epiphytic microflora of vegetables, and include members ubiquitous in terrestrial and aquatic environments as well as human foodborne pathogens, assessing the microbiological safety at the time of consumption is more complicated, with more aspects to consider in monitoring (Rajwar et al., 2015). Concomitantly, fecal coliforms/*Escherichia coli* has been suggested as better indicators for contamination in fresh produce production (FAO and WHO, 2019).

Bacterial contamination of fresh produce can occur via various sources during production (contaminated irrigation water, manure-amended soil), processing (cutting, washing, packaging), distribution and sale (Tope et al., 2016; Koutsoumanis et al., 2021). Of interest to the safety of fresh vegetables are the human foodborne pathogenic bacteria often implicated in foodborne disease outbreaks. Typical foodborne pathogens include selected organisms from the Enterobacteriaceae family such as pathogenic *Escherichia coli* and *Salmonella* spp., as well as *Listeria monocytogenes* (Carstens et al., 2019). Soils amended with treated or untreated

animal manure as fertilizers have been reported as a reservoir of pathogenic microorganisms (Gutierrez-Rodriguez and Adhikari, 2018). The crop may additionally become contaminated if the plant surfaces are in direct contact with manure (Alegbeleye et al., 2018). Furthermore, microbial transport from contaminated soil to the produce occur via splashing from water droplets, from both rain and irrigation water (FAO and WHO, 2019; Machado-Moreira et al., 2019). In fact, irrigation water is considered as one of the most important routes of transmission of enteric human pathogens to vegetable crops and much attention has been given to the microbiological safety of water from different sources used to irrigate fresh produce (Jung et al., 2014; Jongman and Korsten, 2017; FAO and WHO, 2019).

Irrigation water used in South African fresh produce production is often severely compromised mainly due to densely populated human settlements close to the surface water sources as well as mining and industry activities (Oberholster and Botha, 2014; du Plessis et al., 2015; Duvenage and Korsten, 2017; Iwu and Okoh, 2019). Moreover, the deteriorating state of South African wastewater treatment plants (WWTPs) contribute to numerous pollution problems such as frequently releasing effluents of inferior quality into receiving rivers used by farmers downstream for irrigation (Herbig, 2019). During processing, microbial cross-contamination opportunities also arise; when vegetables are cut or shredded, exudates containing nutrients are released that support growth of enteric pathogens (Jung et al., 2014; Castro-Ibáñez et al., 2017; FAO and WHO, 2019). Furthermore, wash water of unsatisfactory microbial quality may aid in dissemination of potential pathogens, while unfavourable conditions such as temperature during packaging and storage can contribute to the growth and survival of spoilage and pathogenic microorganisms on the vegetables (Jung et al., 2014). Unhygienically handled vegetables during distribution and sale, especially for produce which are usually consumed raw, adds an additional potential contamination source within fresh produce supply.

Including surveillance of antimicrobial resistance and the genetic determinants from bacteria found on fresh produce in food safety research has become more common (Ben Said et al., 2016; Hölzel et al., 2018; Koutsoumanis et al., 2021). Antimicrobial resistance genes in addition to acquisition of virulence genes increases the pathogenicity of microorganisms and consequently the severity of infection (El-Baky et al., 2020). In addition to the threat of foodborne pathogens on fresh produce, the prevalence and dissemination of antibiotic-resistant potential pathogenic bacteria on these products are therefore also regarded as an emerging public-health concern (van Hoek et al., 2015; Rico and Falomir, 2020; Koutsoumanis et al., 2021).

Antimicrobial resistance is recognised as a global health challenge. The increasing emergence and spread of drug-resistant pathogens and bacteria acquiring new resistance mechanisms threaten treatment options upon human infection [World Health Organisation (WHO), 2015]. Three groups of Gram-negative bacteria have been identified as critical antimicrobial resistance-related threats globally i.e. i) carbapenem-resistant *Acinetobacter baumannii,* ii) carbapenem-resistant *Pseudomonas aeruginosa*, and iii) carbapenem- and 3rd generation cephalosporin resistant Enterobacterales[1], including *Klebsiella pneumonia*, *Escherichia coli*, *Enterobacter* spp., *Serratia* spp., *Proteus* spp., *Providencia* spp, and *Morganella* spp. (WHO, 2017). The most important within fresh produce and the production environment is the 3rd generation cephalosporin- and carbapenemase resistant members of the Enterobacteriaceae family, as they also occur naturally in these environments (WHO, 2017). An increasing number

[1] A taxonomy change was adopted in 2020 to use “Enterobacterales” as the name of a new scientific order. "Enterobacteriaceae” are now one of seven families within the order, with certain members such as *Serratia* spp. now members of the family Yersiniaceae, while *Providencia* spp. and *Morganella* spp. are members of the family Morganellaceae. This thesis however presents the data according to the previous classification where the order “Enterobacteriales” had a single Enterobacteriaceae family.

of antibiotic-resistant Enterobacteriaceae strains are being detected worldwide, including multidrug-resistant human pathogenic bacteria and their genetic determinants in clinical, food animal, and environmental settings (Kocsis and Szabó, 2013; Iredell et al., 2016; Jones-Dias et al., 2016b; Koutsoumanis et al., 2021). In SA, significant infection outbreaks caused by antimicrobial resistant pathogens have previously included extended-spectrum β-lactamase (ESBL)- producing *Klebsiella pneumoniae* and carbapenemase-producing Enterobacteriaceae in clinical settings (Ekwanzala et al., 2018; Essel et al., 2020). A recent review reported that *Salmonella enterica, E. coli* and *Shigella* are the highest occurring antimicrobial resistant foodborne pathogens in many countries including SA, the U.S and the UK, with the overall number of reported antimicrobial resistance cases in foodborne pathogens generally rising (Yang et al., 2020). Internationally, the need for surveillance of antimicrobial resistance is well recognised (WHO, 2015).

This project aimed to determine the prevalence, dissemination and characteristics of antimicrobial-resistant potential pathogenic bacteria from a food safety perspective in fresh produce production systems and retail. The dual economy system in South Africa (SA) poses additional challenges in terms of microbiological safety and prevalence of antimicrobial resistant pathogenic bacteria on fresh produce sold at informal markets, compared to those sold at formal commercial retailers.

The following objectives were identified:

1. To determine and select vegetables commonly consumed in the formal and informal sector and measure microbial contamination and potential presence of foodborne pathogens.

2. To determine the prevalence of multidrug resistant Enterobacteriaceae, focussing on extended-spectrum β-lactamase production, of isolates from fresh produce sold in formal and informal markets in Gauteng Province.
3. To evaluate the microbiological quality of irrigation water and irrigated spinach from farming, to the packhouse, processing and retail stage and determine the sources of contamination throughout selected commercial supply chains in Gauteng Province.
4. To identify and determine the occurrence, dissemination and characteristics of antimicrobial resistant potential human pathogenic bacteria in the irrigation water and associated spinach from selected commercial farms in Gauteng Province.
5. To compare and link genetic information of environmental isolates from spinach supply chains to potential human pathogenic bacteria using whole genome sequencing (WGS) analysis.

The **first hypothesis** was set as occurrence of antimicrobial resistant Enterobacteriaceae is higher and microbiological safety parameters unsatisfactory for fresh produce sold in the informal compared to formal markets. To test this hypothesis, fresh vegetables that form part of a typical South African food basket were analysed from formal and informal markets. The analysis included indicator bacteria levels (coliforms, *E. coli* and Enterobacteriaceae), foodborne pathogens previously associated with produce-related foodborne disease outbreaks (*E. coli*, *Salmonella* spp., and *L. monocytogenes*) and determining the presence of ESBL/AmpC-producing Enterobacteriaceae. This research question has been addressed in Chapter 3 and Chapter 4 and has been published in Journal of Food Science (Vol 86, pages 161 – 168; doi:10.1111/1750-3841.15534) and Foodborne Pathogens and Disease (Vol 16, pages 421 – 427; doi:10.1089/fpd.2018.2558), respectively.

The **second hypothesis** was that microbiological quality of irrigation water contributes towards the presence and persistence of antimicrobial-resistant bacteria in the spinach production system. Three commercial spinach supply chains that included wholesale and on-farm processing and distribution were investigated and addressed in Chapter 5 and Chapter 6. The three spinach supply chains represented two different production scenarios, where either river or borehole water was used for irrigation.

Analysis included the same microbiological parameters described in Chapter 3, with additional source-tracking of antimicrobial-resistant generic *E. coli* described in Chapter 5 and phenotypic and genotypic characterisation of ESBL-producing Enterobacteriaceae isolated throughout the supply chains addressed in Chapter 6. Chapter 5 has been published in the Journal of Applied Microbiology (doi: 10.1111/jam.15357) and Chapter 6 has been published in Frontiers in Microbiology (Vol 11, pages 1-10; doi:10.3389/fmicb.2020.00638).

The **third hypothesis** was that clinically relevant antibiotic resistance genes are present in environmental Enterobacteriaceae from commercial spinach production environments. Enterobacteriaceae are ubiquitous in human, animal and evironmentl ecosystems, with the ability to exchange antimicrobial resistant genes through mobile genetic elements. Thus, understanding the dynamics of antimicrobial resistance in different sectors are essential for mitigation strategies. To test the third hypothesis, selected ESBL/AmpC-producing Enterobacteriaceae isolates from water and spinach from different points throughout spinach production were characterised using whole-genome sequencing. This research question has been addressed in Chapter 7 and has been published in Frontiers in Microbiology (Volume 12, doi:10.3389/fmicb.2021.734649).

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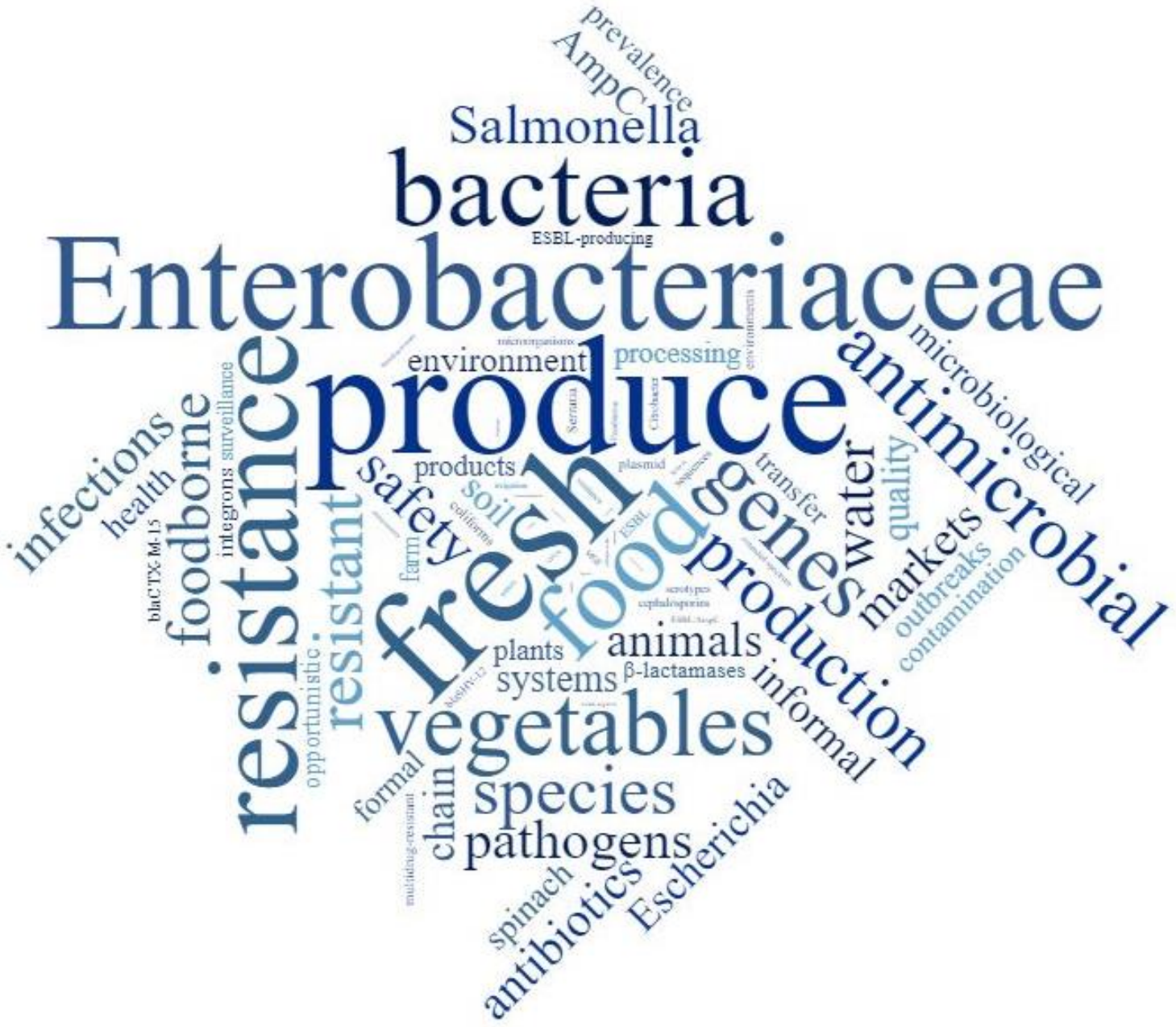
Chapter 2

"The food that you eat can be either the safest and most powerful form of medicine or the slowest form of poison." -*Ann Wigmore*



Literature Review

Abstract

The significance of Enterobacteriaceae in agricultural as well as clinical environments are widely documented. Members of the Enterobacteriaceae family include species that naturally occur in water, soil and plants, as well as foodborne pathogens such as diarrheagenic *Escherichia coli* and *Salmonella enterica*. In SA, fresh produce is sold in a dualistic formal and informal sector, however, surveillance of the microbiological safety of retailed fresh produce is limited. Furthermore, these microorganisms have effective mechanisms to facilitate antimicrobial resistance gene transfer and expression of the acquired genes. With water being a known reservoir of antimicrobial resistance genes, the use of contaminated irrigation water on fresh produce is a potential health threat. Moreover, the prevalence of multidrug resistant bacteria on fresh produce to be consumed raw poses an additional threat to human health. Therefore, the purpose of this review was to assess the relevance of Enterobacteriaceae in fresh produce production and to provide an overview of its associated safety status and antimicrobial resistance levels both in the formal and informal markets. This review provides a critical overview of microbiological quality of fresh vegetables including leafy greens, tomatoes, cucumbers, carrots, green beans and peppers, specifically focusing on foodborne pathogens from the Enterobacteriaceae family (*Salmonella enterica* and pathogenic *E. coli*) as well as *Listeria monocytogenes* which have internationally been implicated in fresh produce related foodborne illness outbreaks. Furthermore, a critical overview of available information on prevalence and characterisation of extended-spectrum β-lactamase (ESBL)- producing Enterobacteriaceae on fresh produce from farm to retail is provided. These studies revealed that numerous Enterobacteriaceae species (both commensal and pathogenic) harbour resistance genes of clinical significance, highlighting the importance of an in-depth study for the

prevalence and characterisation of ESBL-producing Enterobacteriaceae in fresh produce production systems in SA.

2.1 Introduction

Fresh produce have been reported to be carriers and reservoirs of antimicrobial-resistant bacteria, both pathogenic and commensal (Nüesch-Inderbinen et al., 2015; Koutsoumanis et al., 2021). Fresh produce harbouring extended-spectrum β-lactamase (ESBL)- producing Enterobacteriaceae may pose a risk to human health since it is often consumed raw without any additional washing or cooking step (Freitag et al., 2018). All environmental, commensal and pathogenic bacteria, including the associated mobile genetic elements are important reservoirs for resistance (von Wintersdorff et al., 2016). The presence of antimicrobial resistant bacteria, throughout fresh produce supply chains therefore play an important role in the dissemination of antimicrobial resistance among indigenous environmental and pathogenic bacteria (Blaak et al., 2014; Pan and Chu, 2018; Xiang et al., 2018). Antimicrobial resistance is recognised as an important global health problem, with ESBL-producing Enterobacteriaceae being one of the six main antimicrobial resistance health threats (WHO, 2015). If infection by ESBL-producing bacteria occur, treatment options often become difficult as a result of the frequently expanded antimicrobial resistance of the corresponding isolates (Freitag et al., 2018). It is well known that anthropogenic activities are one of the main drivers for high prevalence of antimicrobial resistance genes in the environment (Xiang et al., 2018). Consequently, a global increased incidence of ESBL- and AmpC-producing Enterobacteriaceae in health care and agroecosystems have been reported (Ye et al., 2017).

In addition to antimicrobial resistant bacteria threatening our food system, the introduction of foodborne pathogens onto fresh produce represents an additional threat. Contamination can occur at any stage during production on the farm, in the processing facilities, during distribution

or storage, and at the retail level (Althaus et al., 2012). The significance of Enterobacteriaceae and antimicrobial resistance (including the associated mobile genetic elements) within fresh produce production systems will be discussed in detail as this thesis will seek to investigate the potential link between the water-plant-food-public health interface. Furthermore, the sources of microbial contamination, prevalence of foodborne pathogens, multidrug-resistant- and ESBL/AmpC-producing Enterobacteriaceae, including associated mobile genetic elements, in specific points of fresh produce production systems will be investigated.

2.2 Enterobacteriaceae significance

As Enterobacteriaceae colonise the enteric systems of animals, its transmission to the environment and particularly crop production systems makes it an important microbiological criteria for assessing possible crop contamination related to hygiene and final food safety levels (Rajwar et al., 2015). Enterobacteriaceae also forms part of normal epiphytic microflora of fruits and vegetables making it a more complex system to assess safety at the time of consumption (Rajwar et al., 2015). Further, human and animal pathogenic bacteria are increasingly found to be transmitted through the food chain starting with contaminated fresh produce (Holden et al., 2009). Many of the isolated bacteria from plants are resistant to antibiotics that are frequently used in clinical practice (Markova et al., 2005). The antibiotic classes that are primarily used in SA clinical practice include cephalosporins, fluoroquinolones and aminoglycosides for *Escherichia coli* and *Klebsiella* spp., cephalosporins and fluoroquinolones for *Salmonella* spp., and fluoroquinolones and carbapenems for *Enterobacter* spp., while carbapenems are the only recommended first line therapy for ESBL-producing Gram-negative bacilli (Wasserman et al., 2014). This raises concern with regard to the presence of antibiotic resistant Enterobacteriaceae on fresh produce that is consumed raw and specifically the presence of multidrug-resistant, ESBL-producing Enterobacteriaceae. The ecosystem acts as a reservoir where antimicrobial resistant bacteria can be found in aquatic

systems, faecal matter and soil in the farm environment, as well as plants, and can be transferred from these sources to animals and humans through the food chain (European Food Safety Authority [EFSA] 2011; (Koutsoumanis et al., 2021).

2.2.1 General classification of Enterobacteriaceae

The Enterobacteriaceae family was named according to the organisms' predominant natural habitat i.e. the intestines of warm-blooded animals (from Greek *enteron*, meaning "intestine') (Hardy, 2011). These facultatively anaerobic, non-sporulating rod-shaped bacteria have the ability to colonise, adhere to- and produce various toxins once tissue invasion has occurred (Baylis et al., 2011). Previously, 51 genera and 238 species were acknowledged within the Enterobacteriaceae family, including foodborne pathogens like *Yersinia enterocolitica*, *Salmonella* spp., pathogenic *Escherichia coli*, *Cronobacter* and *Shigella* spp. (Baylis et al., 2011; Octavia and Lan, 2014). The family also included clinically important opportunistic pathogens such as *Serratia* spp., *Citrobacter* spp. and *Klebsiella* spp. (Baylis et al., 2011; Hutchinson, 2014). A taxonomy change was adopted in 2020 to use "Enterobacterales" as the name of a new scientific order. "Enterobacteriaceae" are now one of seven families within the order, with certain members such as *Serratia* spp. now members of the family Yersiniaceae, while *Providencia* spp. and *Morganella* spp. are members of the family Morganellaceae. This thesis however presents the data according to the previous classification where the order "Enterobacteriales" had a single Enterobacteriaceae family.

2.2.2 Ubiquity of Enterobacteriaceae

Members of the Enterobacteriaceae family are widely distributed in humans, animals, and the environment including plants, soil, water and fomites (Baylis et al., 2011). This family is regarded as the most important bacterial family in human medicine as it includes genera and species that cause specific illnesses, and nosocomial infections including wound infections,

meningitis, urinary tract infection, gastroenteritis, pneumonia and septicaemia (Table 2.1) (Doit et al., 2010; Rasheed et al., 2014). Some species are harmless commensals, such as certain strains of *E. coli*, yet other members are pathogenic to humans, animals, plants and/or insects (Table 2.1) (Bari et al., 2011; Baylis et al., 2011; Parija, 2012; Card et al., 2016). Human and animal pathogenic bacteria are increasingly found to be transmitted through the food chain by fresh produce (Holden et al., 2009). Examples include verotoxigenic *E. coli* (VTEC) and *Salmonella* spp., among the most prevalent foodborne pathogens, that are able to enter the food chain at any point (Holden et al., 2009). Additionally, there is growing evidence showing that these pathogenic bacteria do not only contaminate plant surfaces, but may also actively interact with plants and can colonise them as alternative hosts (Holden et al., 2009). Pathogenicity in certain members of the Enterobacteriaceae family can develop as a consequence of gaining virulence-associated genetic material (toxins, colonisation factors) carried on transmissible genetic elements like plasmids, insertion sequences, bacteriophages and transposons (Baylis et al., 2011). As Enterobacteriaceae species are ubiquitous in the environment, water and soil constitutes not only a way of dissemination of pathogenic organisms, but also serve as a route by which resistance genes are introduced in natural bacterial ecosystems (Baquero et al., 2008). Wild animals and insects can also be a source of multidrug-resistant bacteria (Doyle, 2015). Multidrug-resistant bacteria have been detected in cockroaches and house flies at United States (U.S.) swine and Dutch poultry farms (Doyle, 2015; van Hoek et al., 2015). In addition to the presence of antibiotic resistant Enterobacteriaceae in natural water sources, studies have also reported that around 30 different bacterial genera, including *Klebsiella*, *Enterobacter*, *Proteus*, and *Escherichia* have been isolated from recreational and drinking water in India (Ayodhya-Faizabad) and Spain (Seville) (Lechevallier et al., 1988; Kumar et al., 2013; Chiao et al., 2014; Khan et al., 2016).

2.3 Foodborne pathogens and food safety

An increase in demand of ready-to-eat (RTE) minimally processed vegetables also lead to an increase in food safety concerns (de Oliveira et al., 2011b). Indeed, fresh produce have been reported to be a typical vehicle for pathogen carriage and a leading cause of foodborne illness outbreaks (Murray et al., 2017). Over 250 toxins and pathogens are known to be transmitted by food (Choffness et al., 2012). These pathogens include members of the Enterobacteriaceae family such as pathogenic *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica*, and *Cronobacter* spp. (Baylis et al., 2011). *Listeria monocytogenes* is another pathogen often implicated in foodborne disease outbreaks (Zhu et al., 2017).

Escherichia coli is the most widespread facultative anaerobic species found in the gastrointestinal tract of humans, typically colonising infants within a few hours after birth (Kaper, 2005; Baylis et al., 2011). *Escherichia coli* is estimated to kill more than 2 million humans per year through both intra-intestinal and extra-intestinal diseases (Doit et al., 2010; Tenaillon et al., 2010; Centers for Disease Control and Prevention, 2016). The pathotypes of *E. coli* strains can change following the acquisition of new virulence-associated genetic material as certain virulence genes have genetic mobility (Tenaillon et al., 2010; Sarowska et al., 2019). Often, the more infectious pathotypes will have a larger genome when compared to the non-pathogenic *E. coli*, and these diverse virulence factors are usually encoded on chromosomes, plasmids, or bacteriophages (Doit et al., 2010). There are six well described intestinal pathogens that include enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), entero-invasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC) and enteroaggregative *E. coli* (EAEC), with the key virulence factor of EHEC being *stx* (Kaper, 2005; Rojas-Lopez et al., 2018). The serotypes and groups of pathogenic *E. coli* are demarcated by their lipopolysaccharide (O) and flagellar (H) antigens (Tenaillon et al., 2010).

The *Salmonella* genus is divided into 2500 serotypes that cause an extensive diversity of diseases ranging from arthritis to enteritis in humans (Baylis et al., 2011). Two *Salmonella* species are now known; *S. enterica*, which includes serotypes regularly linked to the majority of food-related infections, and *S. bongori*, which is generally connected with reptiles (Baylis et al., 2011). The two dominant serotypes of salmonellosis transmitted from animals to humans are *Salmonella* Enteritidis and *Salmonella* Typhimurium (Public Health England, 2015; Card et al., 2016).

The Gram-positive *Listeria* genus contains four species that are almost exclusively saprophytic (*L. grayi*, *L. innocua*, *L. welshimeri*, and *L. seeligeri*) as well as classified pathogenic species (*L. monocytogenes* and *L. iyanovii*) (Chen and Nightingale, 2013). *Listeria monocytogenes* causes listeriosis with disease symptoms that include mild gastroenteritis as well as more severe disease conditions such as encephalitis, meningitis, septicaemia, abortions, and stillbirths (Zhu et al., 2017). The historical data from the National Outbreak Reporting System (NORS) CDC database (Table 2.2) indicates that only one *Listeria* spp. outbreak was linked to the fresh produce relevant to the current study that have been reported in the U.S. from 1998 to 2017. A substantial amount of literature is available regarding the isolation of *L. monocytogenes* from the relevant fresh produce types (Appendix A, Table 1). Moreover, *L. monocytogenes* have been implicated in a serious listeriosis outbreak in 2011 in the U.S. (CDC FOOD Tool, 2018), linked to contaminated cantaloupe where illness in more than 146 individuals were reported in 28 states leading to at least 30 deaths (Zhu, Gooneratne and Hussain, 2017; CDC FOOD Tool, 2018; CDC National Outbreak Reporting System [NORS], 2020). Recently, SA experienced a serious listeriosis outbreak (between January 2017 and March 2018), with 937 cases and 193 deaths (Thomas et al., 2020). The outbreak was however linked to consumption of deli meats, from two specific food processing companies (Boatemaa et al., 2019; Thomas et al., 2020). Agricultural environments such as water, manure, and soil are part of the natural habitat of

Listeria (Zhu et al., 2017). Further, the ability of *L. monocytogenes* to survive in the food-processing and produce-packing environments and equipment is frequently discussed in scientific literature, emphasising the importance of screening for *Listeria* spp. in fresh produce and processing facilities (Zhu et al., 2017).

Table 2.1: Genera, habitat, optimal growth, pH, and link with foodborne illness causing Enterobacterales documented to be isolated from environmental samples

Genera*	Main habitat*	Optimal growth temperature, pH*	Clinical symptoms/diseases*	Infectious dose (CFU/ml)*	Associated with foodborne illness*
Salmonella	Intestinal tract of humans, animals, birds and insects	35°C – 43°C, 7–7.5	Diversity of diseases ranging from arthritis to enteritis	Varies with the serotype; non-typhoidal salmonellosis: 10^3 bacilli; enteric fever: 10^5 bacilli by ingestion	All are considered pathogenic
Escherichia	Lower intestines of humans, warm blooded animals and birds	37°C, 7	Enteric/diarrhoeal disease, sepsis/meningitis, and urinary tract infections	Varies with the pathotype; E. coli O157:H7: 10^1 – 10^2 other species between 10^6 and 10^{10}	Only the pathogenic strains
Shigella	Intestines of humans and primates	45°C- 47°C, 6 -8	Bacteraemia and seizures, fever, stomach cramps, nausea, vomiting, and flatulence	Very low; 10 - 100 viable cells	All four species
Yersinia	Intestines of humans and animals, also environment	28°C, 7.6	Gastroenteritis; abdominal pain, fever, diarrhea and sometimes vomiting, septicemia	Between 10^4 to 10^6	Some species or strains are pathogenic
Citrobacter	Intestines of humans, animals and birds; also soil, water and sewage	35°C, 6.8 – 7.2	Intra-abdominal sepsis, urinary tract infections, brain abscesses, blood stream infections, and pneumonia and other neonatal infection	10^7	Can be opportunistic
Serratia	Soil, water, plants and rodents	37°C, 5 -9	Respiratory and urinary tract infections, bacteraemia, endocarditis, peritonitis, and cellulitis	Unknown	Can be opportunistic
Hafnia	Intestines of humans, animals and birds; also soil, water and sewage	35°C, 4.9 – 8.25	Infections in the respiratory tract, gastrointestinal tract, urinary tract and colonisation of wounds and devices especially in hospital settings	Unknown	No association
Enterobacter	Intestines of humans, animals and birds; widely distributed in nature, mostly plants	40°C, 7	Endocarditis, bacteraemia, septic arthritis, skin/soft tissue infections, osteomyelitis and lower respiratory tract- urinary tract and intra-abdominal infections	Approximately 1000 cells have been considered infectious	Can be opportunistic
Proteus	Intestines of humans, animals and birds; also soil and polluted water	37°C, 6	Urinary tract infections and kidney infection (pyelonephritis)	Unknown	Can be opportunistic
Klebsiella	Intestines of humans, animals and birds; also soil, water and grain	35°C - 37°C, 7.2	Urinary tract infections, septicaemia, wound infections, pneumonia	Unknown	Can be opportunistic
Kluyvera	Soil, sewage, and water	30°C, 7	Urinary tract infections, sepsis with multiorgan failure	Unknown	Can be opportunistic
Rahnella	Fresh water	37°C, 6	Bacteremia, sepsis, respiratory infection, urinary tract infection, wound infections in immunocompromised patients, and infective Endocarditis in patients with congenital heart disease	Unknown	No association
Erwinia	Mostly plants	28°C, 7.5	Possible causative agent of urinary tract infections	Unknown	No association
Morganella	Intestines of humans, animals, and reptiles	25°C, 5.5	Urinary tract infections, summer diarrhea and nosocomial infections	Unknown	Can be opportunistic

*A compilation of references were used to summarise the information in Table 2.1: (Brisse et al., 2006; Bari et al., 2011; Baylis et al., 2011; Xia et al., 2011; Food and Drug Administration, 2012; Mezzatesta et al., 2012; Nayar et al., 2014; Hadid et al., 2015; Drzewiecka, 2016)

Table 2.2: Details of foodborne disease outbreaks reported by the Centre for Disease Control and Prevention (CDC) National Outbreak Reporting System (NORS) database for produce relevant to the current study

Produce	CDC Foodborne Outbreak Data 1998 - 2017				
	Pathogen	Number of outbreaks	Illnesses	Hospitalisations	Deaths
Pre-packaged leafy greens	*Escherichia*	373	3176	489	14
	Salmonella	757	10656	1781	27
	Listeria	2	5	5	0
Spinach	*Escherichia*	377	3421	570	19
	Salmonella	757	10725	1786	27
	Listeria	2	5	5	0
Lettuce	*Escherichia*	414	5027	760	20
	Salmonella	780	11648	1873	27
	Listeria	3	24	24	1
Cucumber	*Escherichia*	372	3425	456	13
	Salmonella	771	12118	2089	34
	Listeria	2	5	5	0
Tomato	*Escherichia*	317	3136	460	13
	Salmonella	808	15247	2540	33
	Listeria	2	5	5	0
Green beans	*Escherichia*	370	3115	453	13
	Salmonella	759	10786	1834	27
	Listeria	2	5	5	0

2.3.1 Microbiological quality and prevalence of foodborne pathogens on fresh produce

Indicator bacteria are used to provide an indication of poor hygiene, insufficient processing or post-process contamination of foods, as these bacteria are often relatively quick and easy to detect (Baylis et al., 2011). The Enterobacteriaceae family is commonly used as indicator organism by the food industry, with the faecal coliforms, which include a group of lactose-fermenting organisms within this family, used as indication of faecal contamination (Figure 2.1) (Baylis et al., 2011). Internationally, no consensus exists regarding the microbiological standards that apply to RTE minimally processed vegetables (Health Protection Agency, 2009; FSAI, 2016; Fresh Produce Safety Centre Australia & New Zealand [FPSC A-NZ], 2019). Collectively, Enterobacteriaceae have greater resistance to the environment than the coliforms and testing for the entire family

would be more inclusive of the pathogenic bacteria (Baylis et al., 2011). The Health Protection Agency of the United Kingdom (UK) has reported that Enterobacteriaceae counts in RTE foods placed on the market should be $<10^2$ CFU/g to be regarded as satisfactory results, while 10^2 - $\leq 10^4$ CFU/g are borderline and counts $>10^4$ CFU/g are regarded as unsatisfactory, simultaneously, it is reported that these bacteria are not reliable indicators of contamination by faecal pathogens in a food (Health Protection Agency, 2009). Yet, Enterobacteriaceae occur naturally on plants and therefore, these standards to not apply to fresh fruit and vegetables to be eaten raw (Health Protection Agency, 2009). Globally, the trend is to exclude coliforms from specifications as high levels of coliforms are expected in any raw produce (Health Protection Agency, 2009; Health Canada, 2010; CFS, 2014; FSAI, 2016; FPSC A-NZ, 2019). The presence of *E. coli* is used in many countries as a guideline for safety of fresh produce, however, the acceptable limit also differ for the different countries; United Kingdom (20 to 100 CFU/g), Australia (3 to 100 CFU/g), and Canada (100 MPN/g) (Health Protection Agency, 2009; Health Canada, 2010; FSAI, 2016).

A report by the Food and Drug Administration highlighted the routes through which produce can become contaminated (Rajwar et al., 2015). This includes the growing phase through contaminated soil, water, or fertiliser, after harvest through handling and also after purchase during food preparation or inadequate storage, with raw fruits and vegetables carrying the biggest risk of contamination (Rajwar et al., 2015). Depending on seasonal and climatic variation, the numbers of aerobic bacteria present in food may differ in ranges from 10^4 to 10^8 CFU/g with the majority of organisms normally being non-pathogenic to humans (Rajwar et al., 2015). Quantitative methods for detection and enumeration of Enterobacteriaceae are used to prevent or control contamination within food supply chains, as there are often specifications or limits for these bacteria in their products (Baylis et al., 2011; Cardamone et al., 2015). Indicator bacteria such as

coliforms (which falls within the aerobic bacteria group) (Figure 2.2) are consequently used to report the safety assessment of fresh produce throughout different parts of the supply chain.

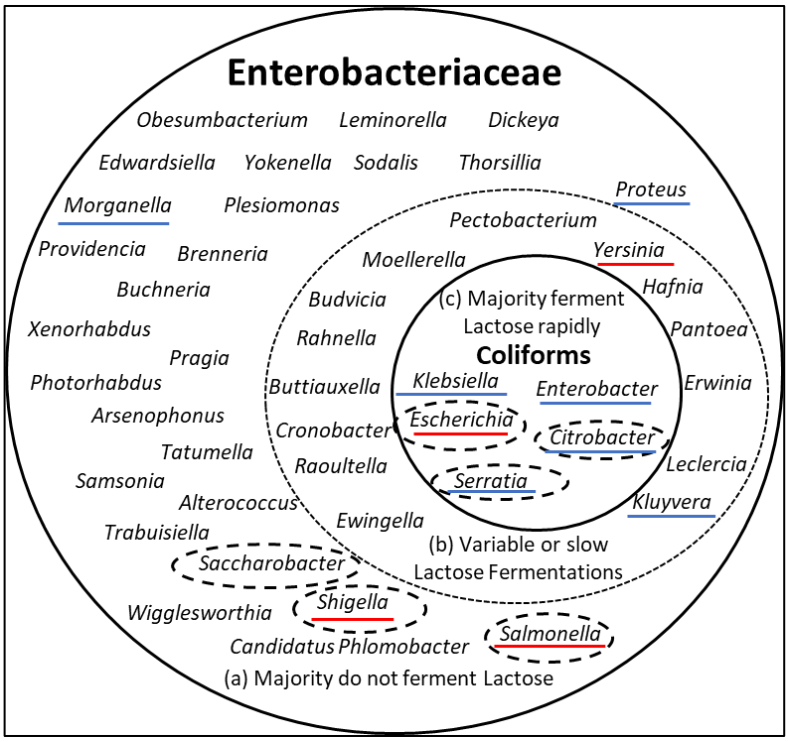


Figure 2.1: The relationship between genera in the coliform group and those within the Enterobacteriaceae family. The dotted circles show genera that include species or strains which commonly cross between two categories (Baylis et al., 2011). Additionally, species with strains often pathogenic to humans are underlined in red and opportunistic pathogens are underlined in blue.

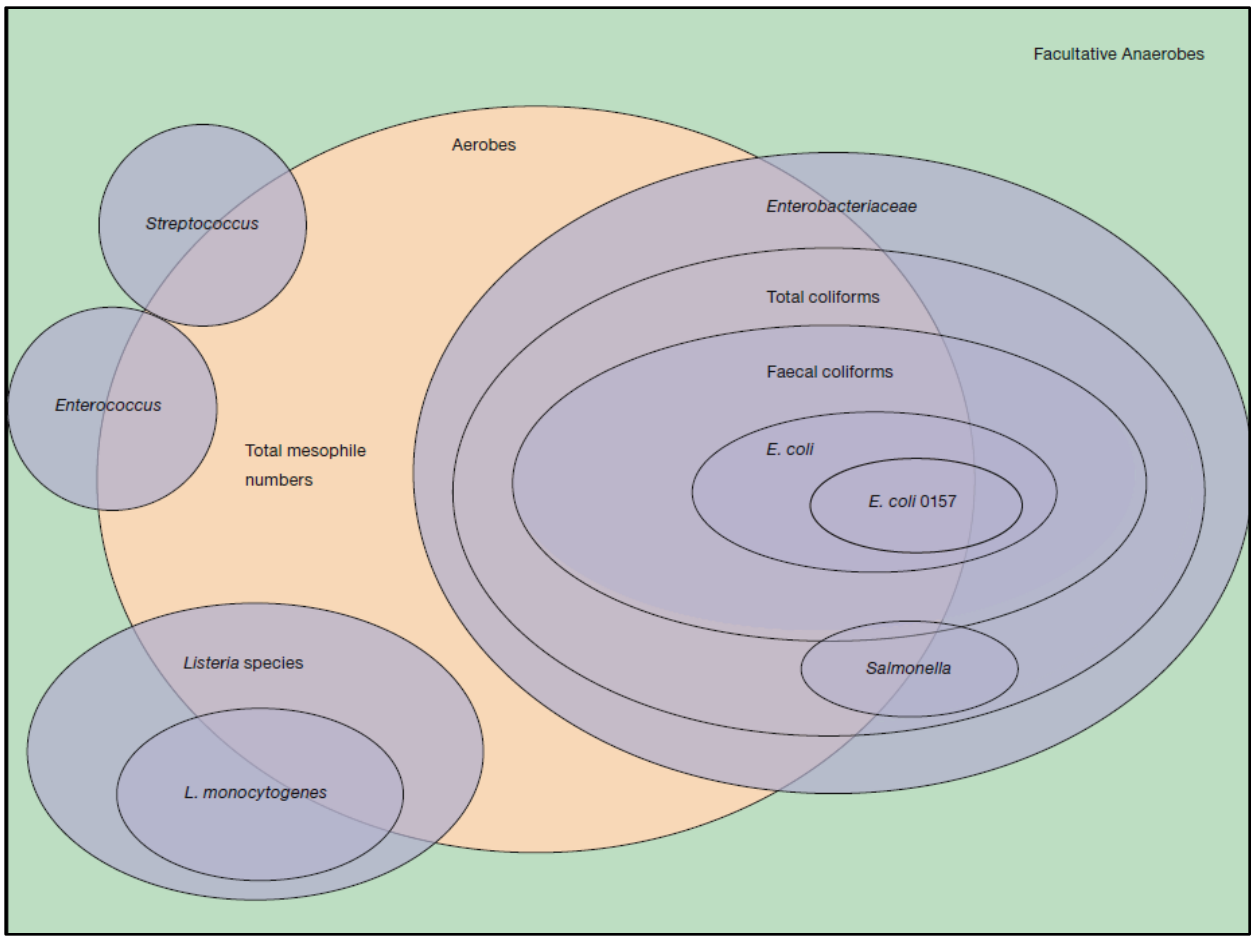


Figure 2.2: The relationships between commonly-encountered bacterial indicators and selected human pathogens (Monaghan et al., 2010).

A literature search was conducted throughout the course of this study to identify potentially relevant publications, prioritizing peer-reviewed journals that reported the microbiological quality of fresh produce and the identification of foodborne pathogens (Figure 2.3). To obtain a comprehensive overview of the microbiological quality of fresh vegetables and the type of crops studied dating back to 2006. A total of 31 publications were found under the specified criteria with the search results indicating the microbiological quality of whole and fresh-cut RTE vegetables that have been studied in different parts of the world at harvest or at a specific point of sale (retailers, informal markets, or farmers' markets) (Figure 2.4, Appendix A, Table A1).

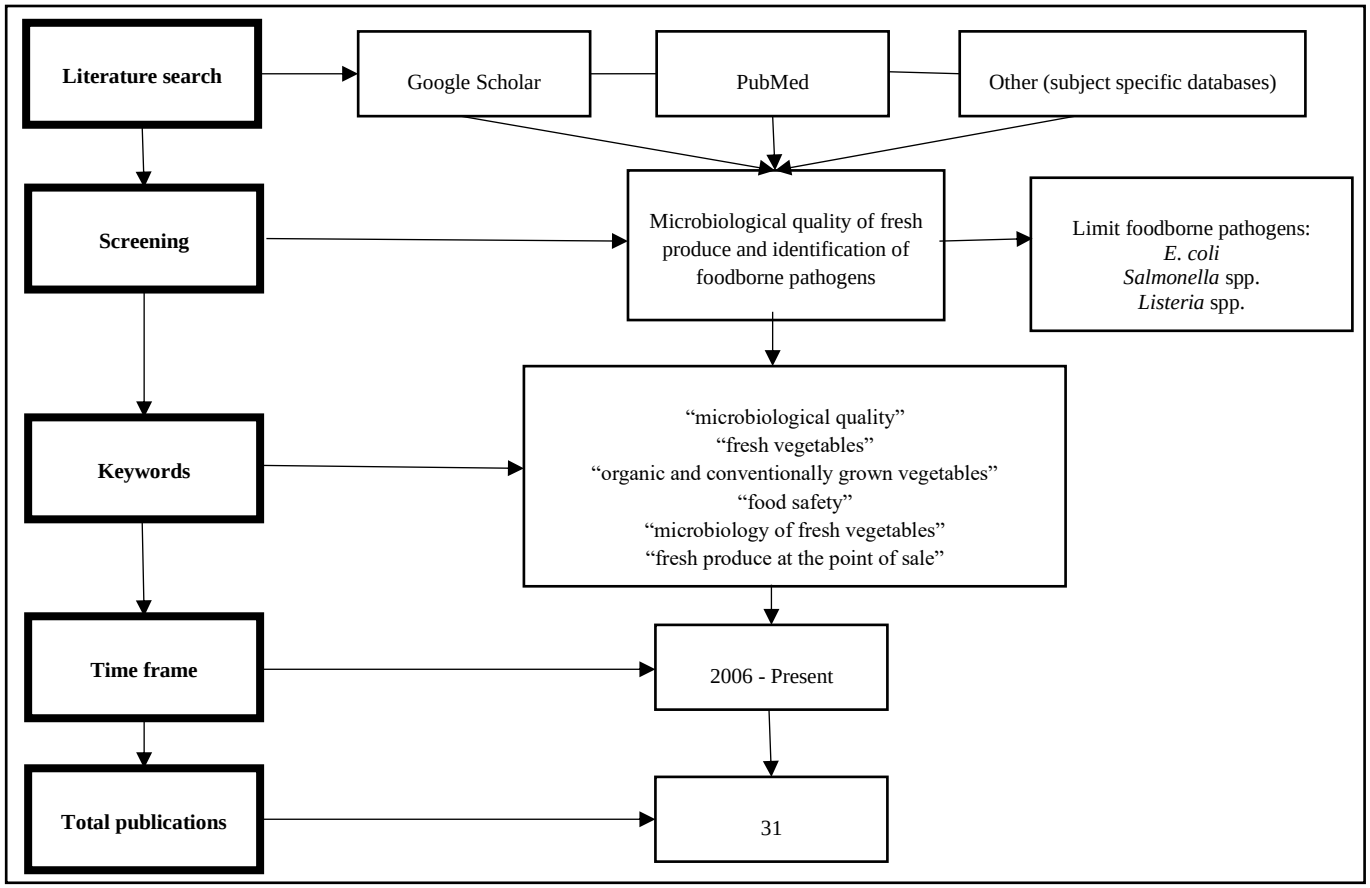


Figure 2.3: Representation of the literature search conducted to identify potentially relevant publications that reported the microbiological quality of fresh produce and the identification of foodborne pathogens.

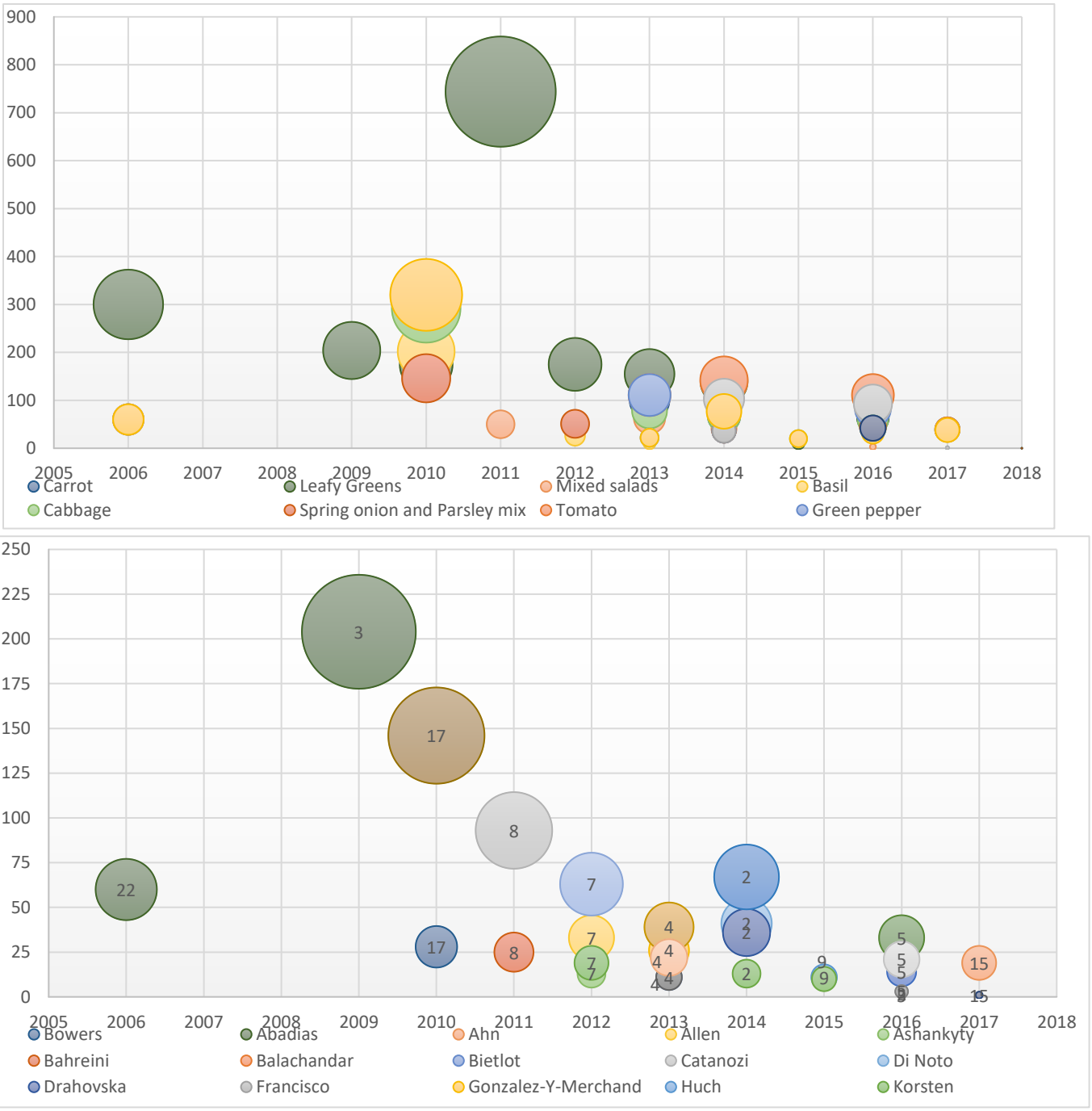


Figure 2.4: Overview of 31 studies dating back to 2006 that focused on the microbiological quality of fresh vegetables. The circle size indicates the citation rate of studies focusing on specific vegetable types (top) and research group leaders (below). The numbers in the circles represent the geographical areas in which the studies were conducted: 1 (Belgium); 2 (Brazil); 3 (British Columbia); 4 (Canada); 5 (Czech Republic); 6 (Germany); 7 (India); 8 (Iran); 9 (Italy); 10 (Malawi); 11 (Malaysia); 12 (Mexico); 13 (Oman); 14 (Pakistan); 15 (Philippines); 16 (Rwanda); 17 (Saudi Arabia); 18 (South Africa); 19 (South Korea); 20 (Spain); 21 (Turkey); 22 (United States of America).

Leafy green vegetables were the predominant (29/31 publications) vegetable types studied, with the most reports (19/31) focusing on lettuce. Other studies with leafy green vegetables included spinach (9/31), cabbage (9/31) and kale (1/31). Several authors have reported on the total aerobic bacteria counts (18/31 publications). The total aerobic counts can however be difficult to use as indicators in fresh produce, as a wide variation in counts have been reported, that lead to additional geometric mean calculations and different microorganisms such as coliforms and faecal streptococci dying off at different rates with ratios changing over time (Monaghan et al., 2010). Therefore, total counts cannot be used as a reliable indicator (Holvoet et al., 2012). In addition, the guidelines for assessing the microbiological safety of RTE foods placed on the market have stated that the total bacterial counts are an indicator of quality, not safety, and therefore cannot directly contribute towards assessment of RTE foods (Health Protection Agency, 2009).

2.3.2 Fresh produce associated with foodborne disease outbreaks

Over a period of 39 years in the U.S., leafy vegetable-associated outbreaks were found to be most prevalent in foodborne disease outbreaks (Herman et al., 2015). With leafy green vegetables forming an important part of a healthy diet, contamination is particularly concerning as these vegetable types are usually consumed raw, thereby excluding any heating step to kill pathogens that might be present (Herman et al., 2015). Further, the availability of ready-to-eat pre-packed bagged salads and green vegetables has increased exponentially (Herman et al., 2015; Arienzo et al., 2020). This follows as changes in packaging, processing, and distribution lead to increased availability and extended shelf-life of leafy green vegetables in the market since the introduction of RTE fresh produce in the early 1980s (Herman et al., 2015). Foodborne disease outbreak data as reported by the CDC in U.S. for specific vegetable types and human pathogenic bacteria implicated mainly *Escherichia*, *Salmonella* and *Listeria* (Table 2.2). Based on the number and

severity of foodborne disease outbreaks associated with the consumption of a specific fresh product, different risk categories have been identified (Callejón et al., 2015). Tomatoes and leafy vegetables (lettuce, rocket, spinach) are regarded as high-risk crops, since they have been linked to a number of foodborne outbreaks world-wide (Callejón et al., 2015). From a microbiological safety perspective, leafy green vegetables are of greatest concern, as they are often consumed raw, or are minimally prepared and therefore have fewer barriers against microbial growth (Mritunjay and Kumar 2015). More recently, not only bacterial and viral pathogens associated with foodborne illness outbreaks involving fresh produce have been mentioned as a concern, but also contamination of parasites such as *Cyclospora*, following major outbreaks in the U.S. from RTE fruit and vegetables (Hadjilouka and Tsaltas, 2020). The impact of pathogenic and spoilage bacteria on parasites' survival within fresh produce supply chains should therefore also be considered in future surveillance studies (Hadjilouka and Tsaltas, 2020).

2.4 Antimicrobial resistance

Due to the widespread (and often inappropriate) use of antibiotics, antimicrobial resistance in different clinical and environmental settings have escalated (Prestinaci et al., 2015). This global spread of antimicrobial resistant organisms have resulted in a major public health challenge, threatening effective prevention and treatment of an increased amount of bacterial infections (Prestinaci et al., 2015; Vikesland et al., 2019). Bacterial resistance to antibiotics occurs through inactivation of the antibiotic by modifying the enzymatic scaffold or enzymatic degradation, by modification of the antibiotic target, adjusting the permeability of the cell membrane, or keeping intracellular concentrations of antibiotics below inhibitory levels through expression of efflux pumps (Vikesland et al., 2019). More recently, mutation of core metabolic genes has been identified as an additional mechanism of antimicrobial resistance in clinical pathogens (Lopatkin

et al., 2021; Wareth et al., 2021). The mobility of antimicrobial resistance genes and the tendency of these genes to spread between different reservoirs including people, animals and the environment further aids in this complex challenge (Vikesland et al., 2019).

2.4.1 Antibiotics mechanisms of action

Antibiotics are grouped according to the specific mechanism of action that includes injury to bacterial cell membranes, the cell wall, inhibition of metabolic biological compounds synthesis, inhibition of nucleic acid synthesis and inhibition of protein synthesis (Shaikh et al., 2015; Kirmusaoglu et al., 2019). Across these groups, ten major classes are currently in use (Vikesland et al., 2019). Beta-lactam (penicillins, cephalosporins, monobactams, and carbapenems) and polypeptide antibiotics which function by inhibiting cell wall synthesis, quinolones and metronidazole which inhibit DNA synthesis, chloramphenicol and tetracyclines which inhibit protein synthesis and sulphonamides that uses competitive inhibition as the mode of action, have all been well documented (Byarugaba, 2009; Kapoor et al., 2017).

2.4.2 β-lactam antibiotics and beta-lactamases

β-lactam antibiotics are the most diverse and most commonly used antibiotics in clinical settings (Shaikh et al., 2015). These antibiotics contain a β-lactam ring that inactivates a set of transpeptidases, also known as penicillin-binding proteins (PBPs), that are usually responsible for catalysis of the final cross-linking reactions of peptidoglycan synthesis in bacteria (Capita and Alonso-Calleja, 2013; Shaikh et al., 2015). This may occur as the β-lactam antibiotics are able to covalently bind to the active site of PBPs, thereby forming a linkage between parallel NAG-NAM strands, leading to interference with peptidoglycan synthesis and resulting in cell death (Katzung et al., 2012; Madigan et al., 2012).

Within bacterial populations, certain bacteria are resistant to β-lactam antibiotics, with the most significant β-lactam resistance mechanism in Enterobacteriaceae consisting of production of β-lactamases (Östholm, 2014; Shaikh et al., 2015). β-lactamases are enzymes encoded by genes either chromosomally located or carried in plasmids (Bush and Bradford, 2016). The β-lactamases work by hydrolysing the peptide bond of the characteristic four-membered beta-lactam ring (Byarugaba, 2009; Bush and Bradford, 2016). Two general schemes are commonly used to classify β-lactamases; the Bush-Jacoby-Medeiros functional classification (Bush et al., 1995) and the Ambler molecular classification (Ambler, 1980). The Ambler molecular classification system classifies β-lactamases into four classes according to the enzyme protein homology (Table 2.3), while the Bush-Jacoby-Medeiros classification scheme is based on functional properties of enzymes (Shaikh et al., 2015). For the purpose of this study, the Ambler molecular classification system will be used for further discussion.

Table 2.3: Classification of beta-lactamases

Beta-lactamases in Enterobacteriaceae					
Ambler class	**Class**	**Subgroups**	**Number of enzymes[f]**	**Phenotypic test**	**Hydrolytic activity against**
A	Penicillinases	Broad-spectrum TEM-1, TEM-2, SHV-1	133	Inhibited by clavulanic acid	Penicillins
	Cephalosporinases $ESBL_A$[a]	TEM-ESBLs SHV-ESBLs CTX-M	200 90	Inhibited by clavulanic acid	Penicillins Cephalosporins
	Carbapenemases $ESBL_{CARBA-A}$[b]	KPC	4	Synergy with boric acid	Penicillins Cephalosporins Carbapenems
B	Carbapenemases $ESBL_{CARBA-B}$[c]	Metallo-beta-lactamases NDM, VIM, IMP	24	Synergy with dipicolinic acid/EDTA	Penicillins Cephalosporins Carbapenems
C	Cephalosporinases non-ESBL	Chromosomal AmpC			
	Cephalosporinases $ESBL_M$[d]	Plasmid-mediated AmpC, CIT (CMY variants), MOX, FOX, DHA, ACC, EBC,	51	Inhibited by cloxacillin	Penicillins Cephalosporins
D	Carbapenemases $ESBL_{CARBA-D}$[e]	OXA-ESBL OXA-48 like	9	Timocillin MIC>32 mg/L	Penicillins Carbepenems

[a]Classification according to Giske et al. 2009; these are often referred to as "classic" ESBLs; [b]Classification according to Giske et al. 2009; $ESBL_{CARBA-A}$ consists mainly of *Klebsiella pneumoniae* carbapenemase (KPC); [c]Classification according to Giske et al. 2009; $ESBL_{CARBA-B}$ are metallo-beta-lactamases (MBL); [d]Classification according to Giske *et al*. 2009; ESBL-M consists of some OXA-ESBLs and AmpC cephalosporinases, which are plasmid- mediated; [e] Classification according to Giske et al. 2009; $ESBL_{CARBA-D}$ is mainly OXA-48-like enzymes; [f](Ghafourian et al., 2015; Bush and Bradford, 2019).

2.4.3 Extended-spectrum-beta-lactamases

The Ambler Class A enzymes are harboured by plasmids and could thus easily be transmitted into different bacterial cells, leading to rapid resistance (Ghafourian et al., 2015). The main enzymes within this class are the TEM and SHV enzymes, with TEM-1 first identified in 1965 in the Enterobacteriaceae family (Ghafourian et al., 2015; Bush and Bradford, 2019). Class A enzymes hydrolyses ampicillin and first, second and third generation cephalosporins, and the extended spectrum beta-lactamases (ESBLs) are also categorised within this class (Ghafourian et al., 2015).

As a phenotypic test for detection of $ESBL_A$, inhibition by clavulanic acid is used, as this has been demonstrated *in vitro* (Östholm, 2014).

Among the classic ESBLs, the enzymes most commonly found include the TEM, SHV, and CTX-M enzymes (Tooke et al., 2019). The variations among these enzymes are diverse with more than 200 TEM and SHV alternates documented (Östholm, 2014). More than 90 different enzymes within the CTX-M type has been described and categorised into five different clusters based on similarities in the amino-acid sequence level(Tooke et al., 2019). The CTX-M type clusters include CTX-M-1, CTX-M-2, CTX-M-9, and CTX-M-25 and are plasmid mediated (Östholm, 2014). The miscellaneous group of ESBLs ($ESBL_M$) include certain OXA-ESBLs as well as plasmid-mediated AmpC cephalosporinases (Östholm, 2014). The $ESBL_{CARBA}$ group consists of carbapenemases which confer resistance to all beta-lactam antibiotics (Östholm, 2014; Tooke et al., 2019).

2.5 Antimicrobial resistant Enterobacteriaceae

Studies have shown a strong link between the occurrence of antibiotic resistance and the composition of the gut microbiome (Tenaillon et al., 2010). Moreover, a link between the resistance patterns of enteric bacteria and the incidence of bacterial disease originating from clinical settings have been reported (Henriksen et al., 2019). An increasing number of antibiotic resistant Enterobacteriaceae strains are detected worldwide, including multidrug-resistant (MDR) human pathogenic bacteria and their genetic determinants in both clinical and environmental settings (Kocsis and Szabó, 2013; Iredell et al., 2016; Jones-Dias et al., 2016b). Important mechanisms of antibiotic resistance in Enterobacteriaceae include porin deficiencies or alterations to reduce antibiotic access and efflux pumps that may actively transport antibiotics out of the cell (Iredell et al., 2016). Additionally, Enterobacteriaceae have β-lactamases acting in the periplasmic

space to hydrolyse β-lactam antibiotics and thereby prevent disruption of the cell wall, as well as intracellular enzymes that alter antibiotics (Iredell et al., 2016).

Enterobacteriaceae are adapted to sharing genetic material through "mobile" resistance genes, as mobile genetic elements with different characteristics can acquire resistance genes from chromosomes and move them between DNA molecules, leading to a much more important resistance mechanism than mutations in chromosomal genes that may also contribute to antibiotic resistance (Partridge, 2015). In human and veterinary medicine, the widespread use of antibiotics is thought to have led to high environmental antibiotic exposure thereby causing ample opportunity for selection of antibiotic resistance in commensal microbiota (Tenaillon et al., 2010). Indeed, dissemination of ESBL-producing Enterobacteriaceae has been identified as one of the six main antibiotic resistance related health risks globally (WHO, 2015). Certain resistance genes are present in the chromosomes of environmental bacteria (Nikaido, 2009). The primary habitat of other Enterobacteriaceae such as *Serratia* spp., *Rahnella* spp. and *Kluyvera* spp. are soil and water and these species are natural carriers of ESBL genes (van Hoek et al., 2015). As an example, presence of the AmpC gene in environmental genera of Enterobacteriaceae such as *Serratia*, *Proteus*, and *Enterobacter* have been reported (Nikaido, 2009). Further, the exclusive animal symbiont *E. coli* lacks the induction mechanism on the AmpC gene and the pathogenic *Salmonella* spp. lacks the AmpC gene entirely (Nikaido, 2009). Studies have however shown that in a random collection of soil-dwelling strains of *Streptomyces* spp. and their relative species, 60% - 100% were resistant to several antibiotics, which suggested presence of antibiotic resistant genes in abundance in this habitat (Nikaido, 2009). However, it should also be noted that the *Streptomyces* genus is a unique subgroup of actinomycetes bacteria and the most prolific antibiotic producers (Kong et al., 2019). Mezzatesta et al. (2012) reported that most isolates of the *E. cloacae* complex

are capable of overproducing AmpC β-lactamases by derepression of a chromosomal gene, or by the acquisition of a transferable AmpC gene on plasmids or other mobile genetic elements. These isolates are intrinsically resistant to first-generation cephalosporins, ampicillin, amoxicillin, amoxicillin-clavulanate and cefoxitin as a result of production of constitutive AmpC, but are susceptible to chloramphenicol, aminoglycosides, tetracyclines, and carbapenems (Mezzatesta et al., 2012).

Plasmid-mediated AmpC strains are distinguished from chromosomal strains because, barring a few exceptions, the expression of the genes are not inducible (Mezzatesta et al., 2012). The AmpC plasmid-mediated strains pose a problem as the derepression of this enzyme is increasingly frequent among clinical isolates, leading to resistance to third-generation cephalosporins (3GC) which are not inhibited by common β-lactamases such as clavulanate, but by boronic acid and/or cloxacillin instead (Mezzatesta et al., 2012). Bacterial species that carry genes expressing ESBLs have been identified as being common inhabitants of the human digestive tract and fresh produce is a possible reservoir of these bacteria (Overdevest et al., 2011). Transfer of multidrug-resistant Enterobacteriaceae onto fresh produce occurs through the use of contaminated irrigation water or during production via animal manure (van Hoek et al., 2015).

2.5.1 Prevalence of antimicrobial resistant Enterobacteriaceae in vegetables

Although most fresh vegetables carry non-pathogenic epiphytic microorganisms, contamination at the farming sites may also arise, as different types of soil treatments such as organic fertilisers that may include sewage sludge and manure are used, as well as the use of contaminated irrigation water and the ability of pathogens to persist and proliferate in vegetables (Tope et al., 2016; Koutsoumanis et al., 2021). Additionally, antimicrobial resistant bacteria can enter the food chain from the farm environment (Figure 2.5) (Tope et al., 2016; Koutsoumanis et al., 2021). These

resistance genes have the potential for horizontal transfer to other related and non-related species, including the gastro-intestinal tracks of mammals (Tope et al., 2016).

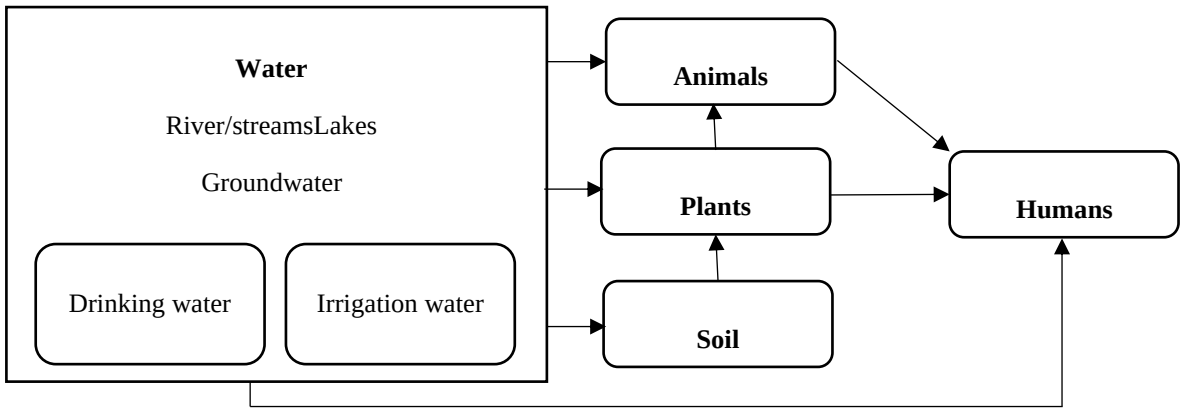


Figure 2.5: Distribution of antimicrobial resistant bacteria from the farm environment.

Antimicrobial resistant Enterobacteriaceae, from farm and retail produce (n =131) were evaluated in Kentucky, USA, with multidrug resistance displayed in 18.2 % and 41.4 % of the isolates from farm produce and supermarket produce, respectively (Tope et al., 2016). Overall, all isolates showed resistance to at least one antibiotic, with Enterobacteriaceae isolated from farm produce that displayed greater resistance to ampicillin (72.7 %) than the isolates from supermarket produce (58.6 %) (Tope et al. 2016). In a similar study, Zurfluh et al. (2015) reported that from 169 vegetable samples, 25.4 % were found to be contaminated with ESBL-producing Enterobacteriaceae of which 78.3 % were multidrug resistant.

The prevalence of ESBL/AmpC- producing Enterobacteriaceae, characterized according to the β-lactamase alleles, isolated from fresh produce from different studies globally were compared in Table 2.4. This comparison indicated that Enterobacteriaceae harbouring *bla*$_{CTX-M}$ variants were the most commonly isolated from fresh produce samples (Blaak et al., 2014; Njage and Buys,

2014; Reuland et al., 2014; Ben Said et al., 2015; Zurfluh et al., 2015; Kim et al., 2015; Nüesch-Inderbinen et al., 2015; Van Hoek et al., 2015; Ye et al., 2017).

Table 2.4: A summary of literature reporting on the prevalence of extended-spectrum- and AmpC β-lactamase producing Enterobacteriaceae isolates including the associated β-lactamase genetic variants from fresh produce samples

Species	Vegetable	β-lactams/β-lactamases					Reference[c]
		ESBL[b]			AmpC	Third generation Cephalosporin (3GC)	
		TEM	SHV	CTX-M			
Citrobacter braakii	Parsnip[a], Carrot,	-	-	blaCTX-M-1	-	-	1
Citrobacter freundii	Blanched celery, Fennel, Radish, Tomato, Raw vegetables, Aragula, Mixed salads	-	blaSHV-12, blaSHV-1	blaCTX-M-15	FOX, CIT	3GC	2,3,4,5,6
Enterobacter amnigenus	Spring onion[a]	-	-	blaCTX-M-15	-	-	1
Enterobacter homaechei	Apricot, Barley	-	-	blaCTX-M-15	-	-	3
Enterobacter cloacae	Lettuce, Cabbage, Mixed salads, Frisee salad, Cucumber, Chopped chives, Bean sprouts, Radish[a], Spinach	-	-	blaCTX-M-15, blaCTX-M-1	DHA-1, MOX, EBC	-	1,7,8,5,6,13
Enterobacter ludwigii	Tomato	-	-	-	MOX	-	6
Escherichia coli	Lettuce, Sprouts, Barley, Parsley, Tomato, Bitter cucumber, Basil leaves, Aragula, Mixed salads, Blanched celery[a], Cucumber	blaTEM-1	blaSHV-12	blaCTX-M-1, blaCTX-M-14, blaCTX-M-15, blaCTX-M-65, blaCTX-M-55	ACC, CIT, DHA	-	1,9,10,3,8,11,5,13
Klebsiella amnigenus	Blanched carrots[a]	-	blaSHV-12	-	-	-	1
Klebsiella pneumoniae	Mixed salads, Sprouts, Bitter cucumber, Garlic chives, Water spinach, Ceylon spinach, Bean sprouts[a]	blaTEM-1	blaSHV-12, blaSHV-11, blaSHV-2, blaSHV-28, blaSHV-1, blaSHV-27, blaSHV-61	blaCTX-M-14, blaCTX-M-15			1,10,8
Kluyvera ascorbata	Diced tomato	-	-	CTX-M Group 2	-	-	7
Serratia fonticola	Coriander, Parsley, Escarole, Cucumber	-	-	-	-	blaFONA-5	12
Rahnella aquatilis	Blanched celery, Blanched carrots, Chicory, Endive, Iceberg lettuce, Radish, Escarole	-	-	-	-	3GC, blaRHAN-2	2,12

[a]The vegetable item was grown organically

[b]TEM-1, SHV-1, -11, -27, -28, and -61 are non ESBL variants

c(1)Reuland et al. 2014, (2)Blaak et al. 2014, (3)Ben Said et al. 2015, (4)Ye et al. 2017, (5)Iseppi et al. 2018, (6)Al-Kharousi et al. 2019, (7)Nuesch-Inderbinen et al. 2015, (8)Zurfluh et al. 2016, (9)Njage & Buys 2014, (10)Kim et al. 2015, (11)Ortega-Paredes et al. 2018, (12)Pintor-Cora et al., 2021, (13)Colosi et al., 2020

2.5.2 Multidrug resistance and dissemination of antimicrobial resistance genes among Enterobacteriaceae

Multidrug-resistant bacteria are defined as bacterial strains exhibiting resistance to three or more classes of antimicrobial substances (Doyle, 2015). Bacteria acquire resistance genes either through mutations or via horizontal gene transfer, the latter being considered as the most important factor contributing towards the high occurrence of antimicrobial resistance (von Wintersdorff et al., 2016). Studies have reported different Enterobacteriaceae strains isolated from various environments having a multidrug resistant phenotype in addition to harbouring ESBL/AmpC encoding genes (Blaak et al., 2014; Ye et al., 2017; An et al., 2018; Freitag et al., 2018).

Dissemination of resistance genes among different strains of bacteria occur as a result of several distinct resistance mechanisms (Deng et al., 2015). These mechanisms include pointmutations, usually occurring at a low frequency, where the bacterial strains acquire multiple genes that each encode resistance to a single drug, within a single cell, or by the increased expression of genes that code for multidrug efflux pumps (Nikaido, 2009; Deng et al., 2015). Further, through acquisition of various resistance genes by means of horizontal gene transfer, which include transduction, transformation and conjugation (White et al., 2001; von Wintersdorff et al., 2016). Diverse multi-resistance regions in chromosomes and plasmids are created through the accumulation of resistance genes around an initial insertion event in a region of DNA which promotes ecological success of the organism (Iredell et al., 2016). Mobile genetic elements (MGE) are predominantly responsible for the capture, accumulation, and dissemination of the antimicrobial resistance genes (Partridge et al., 2018). Mobile genetic elements include among other insertion sequences, transposons, gene cassettes/integrons, as well as plasmids and integrative conjugative elements, that are able to transfer between bacterial cells (Figure 2.6) (Partridge et al., 2018). The interactions

between these different MGE in both Gram-positive and Gram-negative bacteria promote the speedy evolution and diverse multidrug resistance observed in many different environments (Partridge et al., 2018). However, to elaborate in detail on the vast amount of MGE associated with antimicrobial resistance in all bacterial species is beyond the scope of the current study. The importance of the many similarities between the elements' mechanisms as well as some notable differences, such as the significant roles of gene cassettes/integrons in Gram-negative bacteria and small rolling-circle plasmids in Gram-positive bacteria however needs to be noted (Partridge et al., 2018).

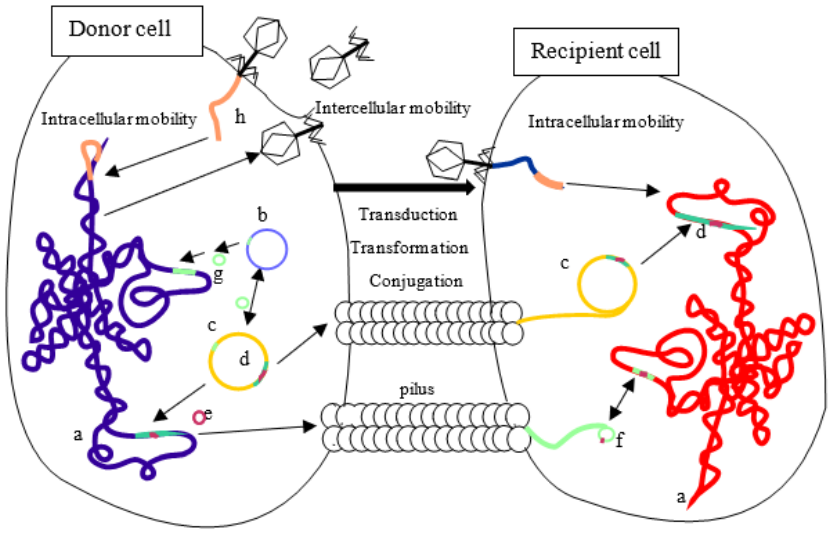


Figure 2.6: Examples of mobile genetic elements involved in intracellular mobility and intercellular (transduction, transformation or conjugation) transfer of antimicrobial resistance genes. (a) Bacterial chromosome where resistance genes can be excised or integrated into new sites, (b) mobilizable plasmid, (c) conjugative plasmid, (d) integron, (e) mobile gene cassette, (f) integrated conjugative element (ICE), (g) transposon, (h) prophage.

Conjugation plasmids and ICEs establish a connection with a recipient cell through a pilus for transfer. Foreign genetic material such as defective genomic islands or a copy of a small plasmid or bacterial chromosome can also be taken up by the recipient cell through transformation.

Temperate phage DNA can insert into the donor bacterial chromosome as a prophage, replicate, lyse the cell and infect a recipient cell through transduction. Within a bacterial cell (intracellular mobility), transposons integrate into new sites on the chromosome or plasmids and integrons also exchange mobile gene cassettes and integrate into the chromosome or plasmids through transposition and non-homologous recombination mechanisms (Frost et al., 2005; Partridge et al., 2018).

2.5.3 Integrons in Enterobacteriaceae

Integrons are defined as DNA elements that mediate the integration of resistance genes through site-specific recombination (Levesque et al., 1995). These DNA elements have been reported to play a critical role in facilitating multidrug resistance in Enterobacteriaceae, regardless of the strain, species or origin (Kaushik et al., 2018). Five classes of integrons have been classified, however, only the first three classes are involved in the spread of resistance genes among Enterobacteriaceae, with class 1 mostly reported (Kaushik et al., 2018). The basic structure or functional platform of class 1 integrons include an *intI* gene, an *attI* site, and a P_c promotor (Figure 2.7) (Kaushik et al., 2018). The integron integrase gene (*intI*) encodes a site-specific recombinase that catalyses recombination between the *attI* recombination site and the 59-base element (59be) recombination site of gene cassettes (Gillings, 2014). Upon recombination, the integron-associated promoter, P_c, regulates the expression of the captured gene cassettes (Gillings, 2014). Gene cassettes contain variable sequences and the level of expression is dependent on the proximity of the gene cassette to the P_c promoter, i.e. the gene cassette that lies closest to the promoter will have a maximum level of expression (Kaushik et al., 2018). The 3'-conserved segment downstream of the gene cassette typically have the *qacEΔ1* and *sul1* resistance genes in class 1 integrons, that encode quaternary ammonium salts and sulphonamide, respectively (Deng et al., 2015). Although

integrons in itself are not mobile, the functional platform linkage to mobile DNA elements such as insertion sequences, transposons or conjugative plasmids thus allow them to serve as important vectors in dissemination of antimicrobial resistance genes (Gillings, 2014; Kaushik et al., 2018).

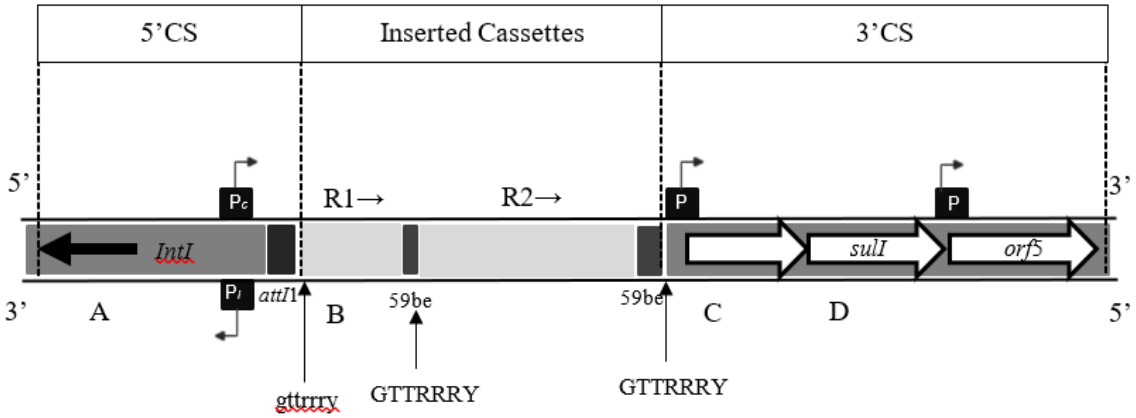


Figure 2.7: Representation of a typical class 1 integron. The arrows indicate the direction of transcription, with location and orientation of promoters shown as P_{int}, P_c and P. A) *IntI* integrase gene and an *attI* site that is recognised by *IntI*. B) Gene cassette that can harbour none, one or many resistance genes (R1, R2) with the sequence GTTRRRY located in the 59be and functions as the crossover point in the integron for integration of the gene cassettes. C) *qacEΔ1* encodes quaternary ammonium resistance and D) *sul1* encodes sulphonamide resistance in the

The rapid development of integrons (especially class 1) associated with multidrug resistant Enterobacteriaceae is well documented (Kaushik et al., 2018). Specifically class 1 integrons have been reported in *E. coli* isolated from clinical samples, animal and water sources, as well as food, from studies dating back to 1973 (Kaushik et al., 2018). Resistance integrons have further been found to be present in *Salmonella* spp., *Serratia* spp., and *K. pneumonia* (Deng et al., 2015), which are all Enterobacteriaceae species previously reported in fresh produce antimicrobial resistance related research (Denis et al., 2016a; Ye et al., 2017a).

2.6 Fresh produce in South Africa

SA is divided into a number of farming regions according to climate, natural vegetation, soil type and farming practices (Goldblatt, 2011). The agricultural activities in the country include intensive crop production and mixed farming in winter rainfall and high summer rainfall areas as well as cattle ranching in the bushveld and sheep farming in more arid regions (Goldblatt, 2011). Fresh produce is cultivated in different regions, leading to processing and distribution facilities found across the country to ensure that the produce is fresh and safe for consumption upon final retail destination (Louw and Jordaan, 2016). In SA, the value of horticultural crops and products (total production during the season valued at the average basic prices received by producers) was reported to be R 332 953 million in 2020. This was an increase of 15.9%, compared to R287 295 million reported in 2019 (Directorate: Statistics and Economic Analysis, 2020). Fresh produce collectively defines raw fruit and vegetables, categorised into ten different subgroups and includes at least 105 different types (Appendix A, Table A2), categorised under horticultural crops and products in the agricultural survey of Statistics SA.

The most recent statistics available (2018) from the Food and Agriculture Organization (FAO) of the United Nations database reported the total production area and estimated tonnes produced in SA for vegetable crops relevant to the current study (Table 2.5) (FAOSTAT, 2020). Although SA is not recognised here as one of the global top growers of spinach (FAOSTAT, 2020), it is well known that local spinach cultivation do occur across different production systems including large-scale commercial, as well as small-scale farms (Jongman and Korsten, 2017). In fact, the popularity of baby spinach has increased globally with the demand for baby spinach overtaking supply in local retail stores (Masufi et al., 2020). Not only commercial scale production, but also small-holder and subsistence farmers contribute to the economy, these are however more difficult

to quantify (GreenCape 2016). Despite the economic importance, production of fresh produce for local consumption is also important in the context of food security.

Table 2.5: Vegetable production per hectare in South Africa as reported by the Food and Agricultural Organisation of the United Nations (FAOSTAT, 2020).

Vegetable crop	Production area (ha)	Estimated tonnes produced
Spinach	No data	No data
Tomatoes	6 521	500 000
Lettuce and chicory	2 462	37 621
Cucumbers and gherkins	1 675	25 133
Green peas	3 704	9 317

2.6.1 Fresh produce supply chains

Supply chains differ in the extent of complexity and time to move the product from production to consumption. For instance, commercial producers are mostly captured in longer chains while informal producers and markets reflect shorter systems. The coordination of the supply chain subsequently plays a vital role in the management of fresh produce, especially in the "big market sales" or formal environment, where the supplier and retailer are often far apart (Su et al., 2014). Whether longer or shorter, the main fact that differentiates these supply chains is the continuous change in volume, product, and quality from the time the raw materials leave the grower to the time the product reaches the consumer (Aung and Chang, 2014). As certain fresh produce types is a class of highly perishable products, longer transportation time could potentially result in more deterioration, influencing the ultimate quality of the product and consequently having a greater impact on retaining market access and consumer trust (Su et al., 2014).

Fresh produce in SA is distributed through formal and informal fresh produce markets, hawkers, export channels and direct sales to wholesalers, processors, or retailers (Figure 2.8). The type or

nature of the fresh produce largely influences the distribution channel that is used for marketing of the product (Louw and Jordaan, 2016). Within a typical market value chain for fresh produce processing facilities, pack houses are often responsible for the handling/cooling and quality standard and packaging aspects of the chain (DAFF 2015).

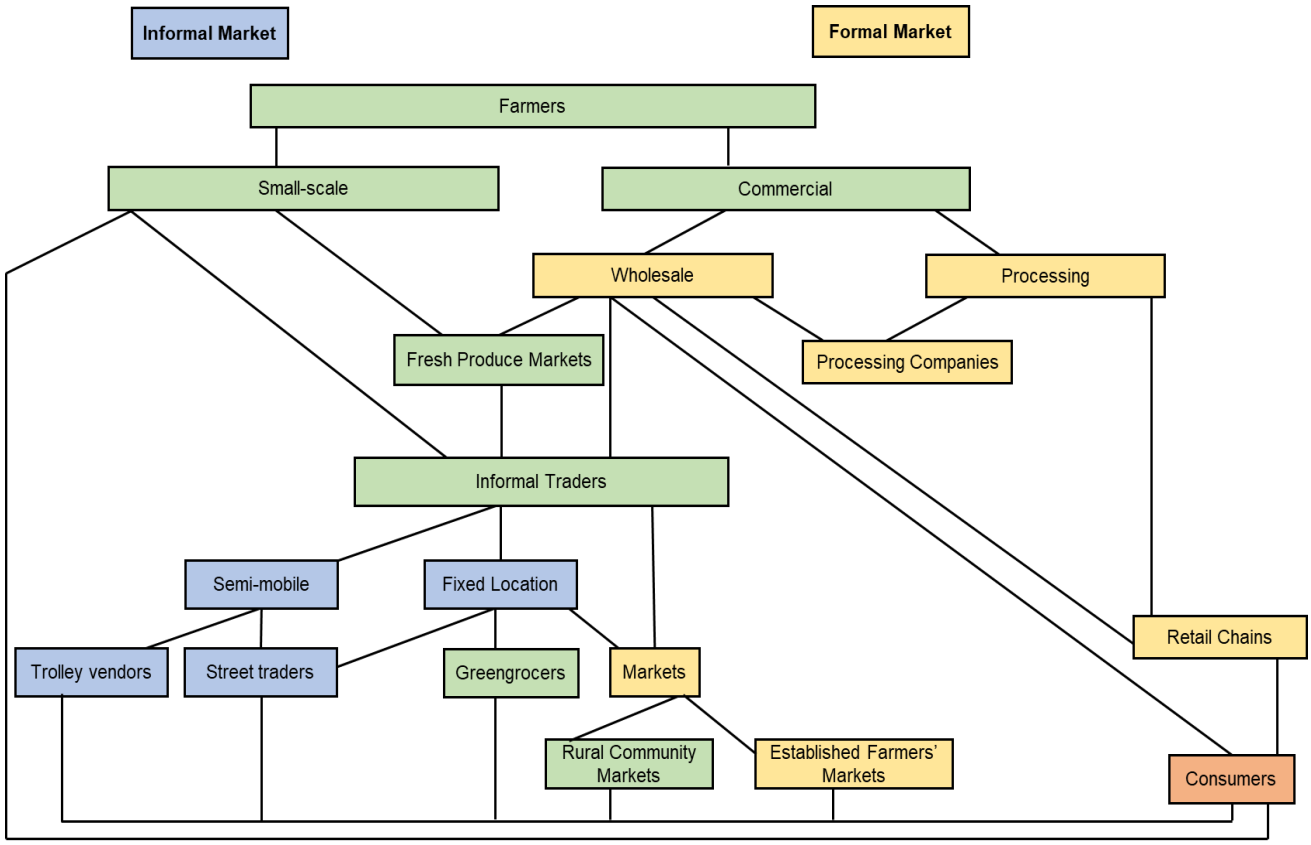


Figure 2.8: Local fresh produce distribution systems in the formal (yellow) and informal (blue) market, with some aspects overlapping (green) between the different markets until fresh produce reaches the consumers (orange).

Processing facilities provide a range of fresh vegetable products that include pre-packed (pillow packs) salad vegetables that contains blends including cos or romaine lettuce, Betavia lettuce, oak leaf lettuce, butter lettuce, red lettuce, baby spinach, broccoli, kale and/or various herbs including rocket, watercress, mizuna, Italian parsley, mint, basil, and rosemary. A web-based search of South African processing facilities indicated that other typical products include pre-cut or chopped

vegetables such as spinach or cabbage, carrots and whole vegetables such as broccoli heads, lettuce heads, cucumbers and tomatoes.

Fresh produce supply chains typically consists of three vertically integrated stages (Shinkfield, 2016). This includes primary production (i.e. growth and harvest), and secondary stages (i.e. processing, washing and packaging), and trade or distribution (i.e. storage, transport and retail) (Shinkfield, 2016). In each of these steps, unique hazards are presented that may influence the possibility of foodborne disease outbreaks as fresh produce are regarded as a high priority in global food safety (Shinkfield, 2016). All hazards, whether chronic or acute, that may make food harmful to the health of the consumer, are referred to as a food safety concern (Aung and Chang, 2014). Food safety is not negotiable and is a global issue, with a worldwide estimated 420 000 human deaths annually as a result of eating contaminated food (United Nations 2021). The food safety responsibility is shared by producers, processors, distributors, retailers, and consumers, as hazards may occur at any of the vertically integrated stages (Aung and Chang, 2014). As a result, supply chains have evolved to obtain effective food safety management systems to bring sufficient and nutritious quality fresh produce to the consumer (Jacxsens et al., 2017).

2.6.2 Fresh produce retail in South Africa

The formal food retail market in SA is dominated by five major commercial retailers. In a review by das Nair & Chisoro (2015) on trends in the supermarket industry in SA, the increase in the number of- and spread of supermarkets locally and to other African countries can be attributed to a number of factors: increasing urbanisation, increased per capita income, increase in number of women working, increased middle class size, lower prices due to economics of scale and scope of products on offer and modernisation of infrastructure. While formal retailers used to mainly procure their fresh produce from municipal markets, key retailers now have central procuring

systems in place, where fresh produce is obtained from a number of preferred suppliers (Louw et al., 2006). In 2003, supermarkets in SA were estimated to have a 55% share of the national food retail market, as opposed to an estimated 10% to 20% in the early 1990s (White, 2011). In commercial supply chains, traceability standards are enforced to ensure that, if an outbreak occurs, the source can be identified (Aung and Chang, 2014; Chhikara et al., 2018). Traceabiltiy is defined as the ability to trace the history, application or location of that which is under consideration according to the ISO 9000 (2015) standards. Information can be recalled in different directions within a chain. Backward traceability or tracing refers to finding the origin and characteristics of a product based on one or several given criteria , while forward traceability, or tracking refers to finding at every point of the supply chainthe locality of products from one or several given criteria (Aung and Chang, 2014; Zhong et al., 2017; Chhikara et al., 2018). In certain supply chains, especially in the informal sector, challenges are found in contamination source-tracking, as products often lack any labelling and distribution records, multiple sources of a certain product at a single point of sale may occur, and complex distribution systems are often followed (Aung and Chang, 2014). The smallholder fresh produce supply chain in SA is characterised by various distribution channels that include farmers' markets, fresh produce markets (FPMs), hawkers, greengrocers, local consumers, and institutional buyers such as government hospitals that farmers use to distribute their produce, depending on demand and accessibility (Louw and Jordaan, 2016).

A farmers' market is commonly defined as a regular event in a town or city when farmers come to sell their fresh produce, eggs, meat, etc, directly to customers (Saili et al., 2007). More specifically, farmer's markets in the UK describe food markets where produce from a defined local area is sold directly to the public and produced by the vendors (Vecchio, 2011). Farmer's markets are often perceived as alternative food networks, providing a link between rural food producers and urban

consumers (Vecchio, 2011). In SA, farmers' markets have gained popularity and is an excellent marketing platform for small scale farmers (van der Heijden and Vink, 2010). These markets form a small part of the fresh produce distribution network, however their popularity have been growing since good quality produce can be bought at a more affordable price when compared to commercial retailers (Vermeulen and Bienabe, 2007). Farmers markets are an excellent example of short food supply chains, which are drivers of sustainabe development as well as food production and contribute to improving the food security status in the country (van der Heijden and Vink, 2010). Although farmers markets play a relatively small role in fresh produce retail, they offer an excellent platform for small scale farmers to sell their produce (van der Heijden and Vink, 2010).

In sub-Saharan Africa, informal sector employment comprises 53% and although the individual incomes of informal workers are often low, cumulatively their activities contribute significantly to gross domestic product (Skinner and Haysom, 2016). Moreover, small-scale farmers contribute substantially to the provision of food in SA and other countries (Hlophe-Ginindza and Mpandeli, 2020). People who are in the low socio-economic status almost solely depend on informal markets due to the location (the market stalls are usually near taxi ranks, industries, pavements and also train stations) (Methvin, 2015). This resulted in a large volume of fresh produce being sold in townships and informal settlements in SA (Charman, 2015; Methvin, 2015). A business will take the shape of either street trading greengrocers, where a stall comprises of a table and shade covering, or mobile trolley vending, where fruit and vegetables are packed in bags and sold from the trolleys (Figure 2.9). Fresh produce sold at the street vendors are bought from home gardens, local small-scale farms, national fresh produce markets (NFPMs), or from formal retailers (Roever and Skinner, 2016). In addition, the informal traders are the main purchasers of fresh produce sold by small scale farmers (Louw, 2008). Therefore, informal markets have the advantage to source

fresh produce without being concerned about the high prices associated with formal supply chains (Louw, 2008). Traceability is typically non-existent in the informal market, as no formal grades and rarely any standard measures are implemented (Ferris et al., 2014). The benefits of this system leads to relatively low levels of postharvest loss, creating an environment for extreme flexibility in value propositions and thereby attracting a wide variety of buyers and suppliers (Ferris et al., 2014). In contrast, the formal market commonly require traceability throughout a supply chain (Ferris et al., 2014). This is implemented by adherence to a series of best practices for the production and handling of food based on food safety standards by each actor in the supply chain (Ferris et al., 2014).

Figure 2.9: Examples of informal fresh produce traders in South Africa. Photo on the left: produce sold at a farmer's market in Pretoria, Gauteng Province, photo in the middle: produce sold at a street vendor in Tembisa, Gauteng; photo on the right: example of a trolley vendor where vendors sell fresh produce in prepacked bags in Tembisa, Gauteng Province.

The most recent report available stated that the vegetable components of a basic food basket includes cabbage, onions, potatoes and tomatoes (NAMC, 2016, 2020). According to Statistics SA, vegetables within the consumer price index (CPI) in all urban areas throughout SA include leaf and stem vegetables, vegetables cultivated for their fruit, root crops, non-starchy bulbs and mushrooms, dried vegetables, other preserved or processed vegetables, and vegetables cultivated for their tubers (Bennet, 2016). The Pietermaritzburg Agency for Community Social Action (PACSA) reported in 2014 that vegetables chosen for the ideal food basket, taking cultural

acceptability, nutritional value and cost into consideration, includes onion, tomato, carrot, spinach, cabbage, green pepper, and butternut (Barnard, 2014). The most common fresh produce sold by street vendors are spinach, potatoes, tomatoes, sweet corn, sweet potatoes, tomatoes, peas, cabbage, beans, onions, lettuce, okra and also other indigenous vegetables/leafy greens which are usually referred as morogo (Mthombeni, 2013). The fresh produce that street vendors usually purchase from the NFPMs include: potatoes, onions and tomatoes with other vegetables and fruits such as citrus, deciduous, and subtropical fruit. In terms of fresh vegetables: carrots, green peas, cabbage, beetroot, green beans, cauliflower, pumpkins, green mealies, and sweet potatoes make up the bulk of the produce (Louw, 2008).

2.7 Conclusion

The Enterobacteriaceae family has significance in fresh produce production systems and food safety. Furthermore, as these microorganisms have effective mechanisms to facilitate antimicrobial resistance gene transfer and expression of the acquired genes, the potential prevalence of multidrug resistant bacteria on fresh produce to be consumed raw poses an additional threat to human health. Worldwide, consumption of fresh fruit and vegetables are increasing for the many health benefits, concurrently, reports on foodborne disease outbreaks associated with fresh produce are also increasing. As contamination could occur during any stage of pre- and post-harvest fresh produce production, the need for effective surveillance for microbiological safety along the entire supply chain, from the farm, throughout processing, up to retail is highlighted. This includes surveillance of antimicrobial resistant bacteria and the potential transfer of the resistant genes along supply chains. The dualistic food market in SA however poses additional challenges for surveillance, as information regarding production and distribution especially in the informal sector, is often limited. Yet, 50 % of the SA population depend on informal markets for

fresh produce supply. This emphasises the need for continuous surveillance of the microbiological safety and prevalence of antimicrobial resistance in fresh produce across all supply sectors in SA. Current surveillance data of the microbiological quality of fresh produce, including the prevalence and genetic determinants of ESBL/AmpC producing Enterobacteriaceae on fresh produce products in SA is limited.

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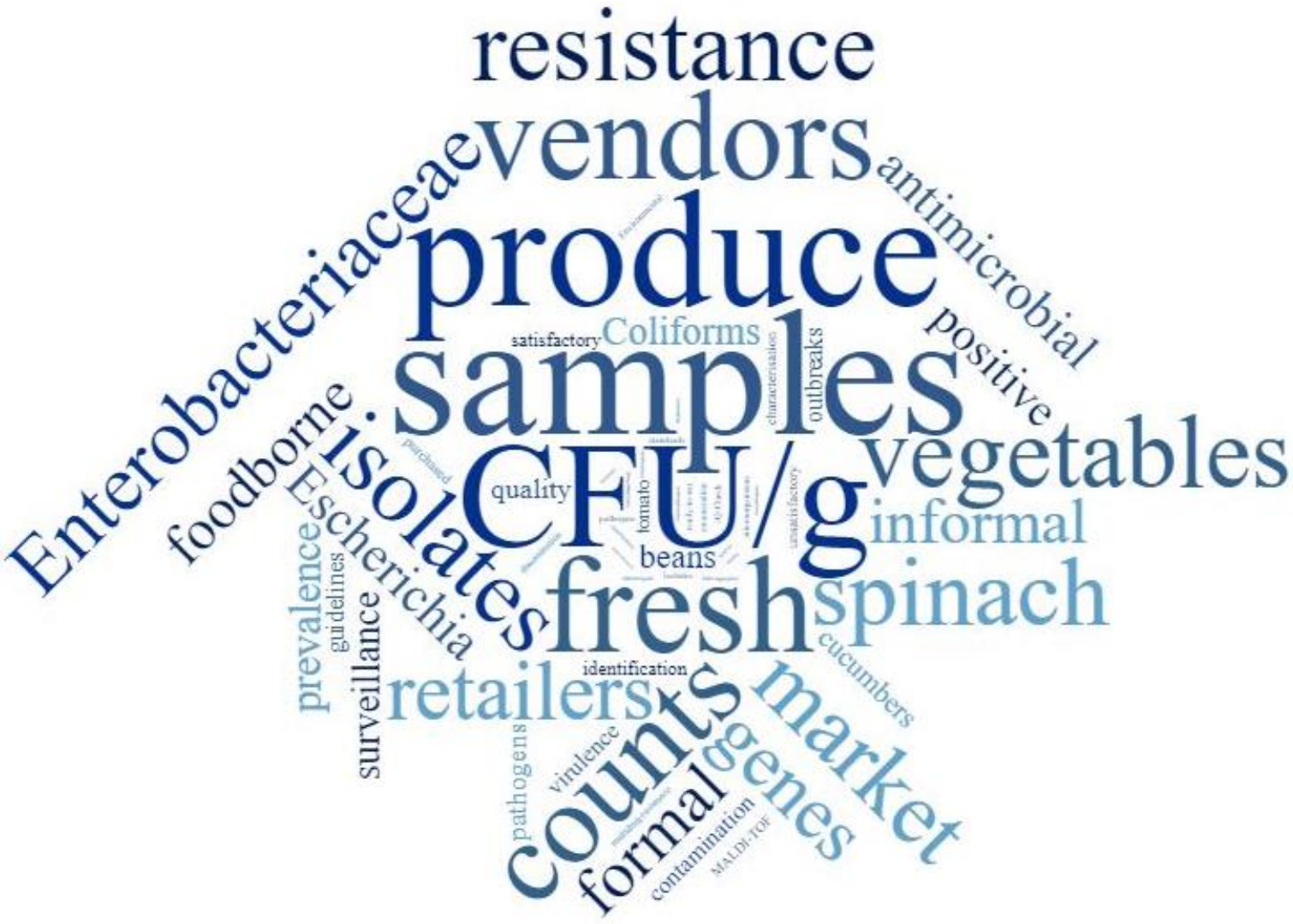
Chapter 3

"Food safety involves everybody in the food chain." -*Mike Johanns*



Fresh Produce at the Point-of-Sale

10 Retailers | 10 Trolley vendors | 10 Street traders | 13 Farmers' market vendors

545 Samples

Microbiological safety and antimicrobial resistance profiles of isolated *Escherichia coli*

Compliant to international standards (*E. coli* ≤ 100 CFU/g):

- 70-94% spinach,
- 90-98% tomatoes,
- 93% lettuce
- 82% cucumbers, and
- 80% green bean samples

14.86% of the samples (n=81) harbored *E. coli* - mostly from leafy green vegetables.

- No *Salmonella* spp. or *Listeria monocytogenes* detected
- No virulence genes (*lt, st, bfpA, eagg, eaeA, stx1, stx2, ipaH*) detected in the *E. coli* isolates (n=67) characterized
- 40.30% *E. coli* isolates were multidrug-resistant

Necessary to consider characterisation of Enterobacteriaceae with expanded spectrum antimicrobial resistance

There is a need for improved food safety practices within the supply chains and identification of fresh produce contamination sources

First study to investigate the microbiological quality (including Enterobacteriaceae enumeration) and occurrence of multidrug resistant generic *E. coli* in fresh vegetables sold at formal and informal markets in Gauteng Province, South Africa

Richter, L., du Plessis, E.M., Duvenage, S., and Korsten, L. (2021).I high prevalence of multidrug resistant *Escherichia coli* isolated from fresh vegetables sold by selected formal and informal traders in the most densely populated Province of South Africa. J. Food Sci. 86, 161–168. doi:10.1111/1750-3841.15534.

Plant to Food

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Prevalence of multidrug resistant *Escherichia coli* isolated from fresh vegetables sold in formal and informal traders in Gauteng Province, South Africa[2]

Abstract

Contaminated fresh produce has increasingly been implicated in foodborne disease outbreaks. As microbiological safety surveillance in South Africa is limited, a total of 545 vegetable samples (spinach, tomato, lettuce, cucumber and green beans) were purchased from retailers, street traders, trolley vendors and farmers' markets. *Escherichia coli*, coliforms and Enterobacteriaceae were enumerated and the prevalence of *E. coli*, *Salmonella* spp. and *Listeria monocytogenes* determined. *Escherichia coli* isolates were characterised phenotypically (antibiotic resistance) and genotypically (diarrheagenic virulence genes). Coliforms, *E. coli* and Enterobacteriaceae counts were mostly not significantly different between formal and informal markets, with exceptions noted on occasion. When compared to international standards, 90-98% tomatoes, 70-94% spinach, 82% cucumbers, 93% lettuce and 80% green bean samples, had satisfactory (100 -1000 CFU/g) *E. coli* counts. Of the 545 vegetable samples analyzed, 14.86% (n=81) harbored *E. coli*, predominantly from leafy green vegetables. Virulence genes (*lt*, *st*, *bfpA*, *eagg*, *eaeA*, *stx1*, *stx2*, *ipaH)* were not detected in the *E. coli* isolates (n=67) characterized, however 40.30% were multidrug-resistant. Resistance to aminoglycosides (neomycin, 73.13%; gentamycin, < 10%), penicillins (ampicillin, 38.81%; amoxicillin, 41.79%; augmentin, < 10%), sulfonamides (cotrimoxazole, 22.39%), tetracycline (19.4%), chloramphenicol (11.94%), cephalosporins

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(cefepime, 34.33%) and carbapenemases (imipenem, < 10%) were observed. This study highlights the need for continued surveillance of multidrug resistant foodborne pathogens in fresh produce retailed formally and informally for potential consumer health risks.

3.1 Introduction

Surveillance of the microbiological quality of fresh produce at retail level have been reported in various countries (de Oliveira et al., 2011a; Ryu et al., 2014; Kuan et al., 2017; Li et al., 2017; Sair et al., 2017; Roth et al., 2018b; Tango et al., 2018), with increasing numbers being associated with fresh produce resulting in foodborne disease outbreaks (Denis et al., 2016). This highlights the need for effective foodborne disease outbreak surveillance and reporting systems in fresh produce supply chains. The South African food market is characterized by dualism; both well-developed, highly sophisticated and regulated formal- as well as the less regulated informal food systems that provide fresh produce to consumers throughout the country (Louw et al., 2006; Skinner and Haysom, 2016). Differences in the production and distribution systems raise the question of possible differences in microbiological quality of the retailed fresh produce (Verraes et al., 2015).

Enterobacteriaceae form part of the indigenous microbiota of vegetables (Blaak et al., 2014). Members of this family, i.e. pathogenic *Escherichia coli* and *Salmonella* spp., have often been associated with foodborne bacterial outbreaks following raw fresh produce consumption (Tope, Hitter, & Patel, 2016). This includes diarrheagenic *E. coli* strains, including enteropathogenic (EPEC), enterotoxigenic (ETEC), enterohaemorrhagic (EHEC), enteroaggregative (EAEC), and enteroinvasive (EIEC) *E. coli* in foodborne disease outbreaks (Aijuka et al., 2018; Canizalez-Roman et al., 2019). In addition to generic *E. coli*, diarrheagenic strains are also found in the intestinal tracts of mammals and are therefore often used as indicators of fecal contamination in fresh produce supply chains (Denis et al., 2016a). Similarly, *Listeria monocytogenes* is

increasingly linked to fresh produce associated foodborne disease outbreaks globally (Zhu et al., 2017), but until recently, rarely reported in South Africa (SA), particularly associated with fresh produce (Kayode et al., 2020).

As fresh produce is often consumed raw or minimally processed, no "kill step" occur, leaving fewer barriers against microbial contamination (Mritunjay and Kumar, 2015). A previous study where the microbial quality of fresh produce sold in SA was investigated, reported that antibiotic resistant *E. coli* occurred in leafy green vegetables sold formally and informally in Johannesburg, SA (du Plessis et al., 2017). The importance of large-scale microbiological surveillance in the formal and informal supply chains were highlighted, focusing attention on the comparative safety levels of food sold in SA. The solitary focus on foodborne pathogen prevalence in the world has expanded in the last decade to include more formal surveillance of antimicrobial resistance (AMR) in microorganisms in agricultural production systems including fresh produce (Ben Said et al., 2016; Blaak et al., 2014; Ye et al., 2017). This follows after the World Health Organization (WHO) highlighted the need for a global AMR surveillance system in various countries (WHO, 2015). It was further reported that members of the Enterobacteriaceae family form part of the priority pathogens for surveillance of AMR (WHO, 2015). Environmental bacteria naturally harbor resistance genes to certain antimicrobials on their chromosomes (Blaak et al., 2014). However, the widespread use of antimicrobials in for example hospital settings and agricultural production (e.g. animal husbandry) has resulted in the selection of multidrug resistant microbes, posing a broader threat to the treatment foodborne diseases (Doyle, 2015). Indeed, serious patient treatment complications may arise if multidrug resistant *E. coli* (or other foodborne pathogens) are ingested, even if no immediate or obvious health outcome arise (O'Flaherty et al., 2019). This follows as

transfer of antibiotic resistant genes to other bacterial species in the human gut may occur, increasing the risk of future antibiotic treatment options (O'Flaherty et al., 2019).

The aim of this study was to determine the microbiological safety (coliforms, *E. coli* and Enterobacteriaceae) and presence of potential human pathogenic bacteria (*E. coli*, *Salmonella* spp. and *L. monocytogenes*) in vegetables sold at formal retailers, informal street- and mobile trolley vendors, and from farmers' markets in the densest urban area in SA. The *E. coli* isolates from vegetables were characterized using phenotypic (antimicrobial resistance) and genotypic (*lt*, *st*, *bfpA*, *eaeA*, *eagg*, *stx1*, *stx2* and *ipaH* virulence genes) analysis.

3.2 Materials and Methods

3.2.1 Sample collection and processing of fresh produce

Ten suppliers in retail and twenty in informal markets (ten street traders and ten mobile trolley vendors) as well as 13 stalls from two farmers' markets in Gauteng Province SA were selected for sampling (Appendix B, Figure 1B). In total, 545 randomly chosen vegetable samples were purchased between September 2017 and May 2018. Depending on availability, spinach (bunches, baby leaves, or minimally processed ready-to-eat (RTE) pillow packs) and tomatoes, from retailers, street traders, trolley vendors and farmers' markets (n=50 from each respective group), were analyzed. In addition, cucumbers (n=45), lettuce (Iceberg lettuce heads or mixed salad leaf RTE pillow packs) (n=50), and green beans (n=50) were also included from the farmers' market vendors. All samples were transported cooled and stored at 4°C until further processing within 24 h.

A 50 g composite sample for each of the respective leafy vegetables were aseptically cut into a sterile polyethylene strainer stomacher bag containing 200 ml buffered peptone water (BPW) (3M,

Johannesburg) in a 1:4 weight to volume ratio (Richter et al., 2019). For the tomatoes and cucumbers (composite samples of at least three from each product), as well as green beans, 150 g samples were each placed into a sterile polyethylene stomacher bag containing 150 ml BPW in a 1:1 weight to volume ratio (Xu et al., 2015). Individual vegetable samples were blended for 5 min at 230 rpm in a Stomacher® 400 Circulator paddle blender (Seward Ltd., London, UK).

3.2.2 Microbiological analysis

To enumerate coliforms and *E. coli*, a tenfold dilution series of each BPW sample mixture was plated in duplicate onto *E. coli*/coliform count plates and incubated for 24 h at 37°C according to the manufacturer's instructions (3M Petrifilm, 3M, St. Paul, Minnesota, USA, ISO method 4832). Enterobacteriaceae were enumerated by plating in duplicate onto Violet Red Bile Glucose (VRBG) agar plates and incubated for 24 h at 37°C (Oxoid, Johannesburg). The remaining sample in BPW was incubated for 24 h at 37°C for detection of *Salmonella* spp. and *E. coli*. After incubation, the samples in BPW were subsequently streaked onto Eosin methylene blue (EMB) media (Oxoid) for the detection of *E. coli*. The presence of *Salmonella* spp. was assessed using the iQ-Check *Salmonella* II Kit AOAC 010803 (BioRad, Johannesburg) according to the manufacturer's instructions. Once positive results were obtained, the sample was streaked onto Xylose lysine deoxycholate (XLD) agar (Biolabs, Johannesburg) and *Salmonella* Brilliance agar (Oxoid) and incubated for 24 h at 37°C. The presence of *Listeria* spp. was assessed by incubating an additional 25 g of each sample in 225 ml Buffered *Listeria* Enrichment Broth (BLEB) (Oxoid) at 30°C for 24 h and subsequently using the iQ-Check *Listeria monocytogenes* II Kit AOAC 010802 (BioRad) according to the manufacturer's instructions. Once positive results were obtained, the sample was streaked onto Agar Listeria Ottavani and Agosti (Biomèrieux SA, France) and Rapid'L.mono agar (BioRad) and incubated for 48 h at 37°C. All presumptive positive *E. coli*, *Salmonella* spp. and *L.*

monocytogenes colonies were isolated and purified. Isolates were identified using matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF) (Bruker, Bremen, Germany) to species level as described by Standing et al., (2013) and AOAC-OMA#2017.09. Briefly, purified strains were transferred in duplicate onto the MALDI-TOF steel polished target plate, overlaid with the α-cyano-4-hydroxycinnamic acid matrix (Bruker, Bremen, Germany) and analyzed using MicroFlex LT MALDI-TOF (Bruker) in conjunction with the Biotyper automation software and library (Bruker) following calibration with a bacterial standard according to the manufacturer's instructions (Bruker). The best organism match score values ranging between 2.30-3.00 were considered reliable for identification at species level, whilst the best organism match score values ranging between 2.00-2.29 were considered reliable for genus level, with probable species identification, and values between 1.70-1.99 were considered as probable genus identification (Appendix B, Table B3).

3.2.3 Antimicrobial susceptibility testing

A total of 67 isolates were selected which included one representative *E. coli* isolate per product type found from each supplier and tested further for antimicrobial resistance or susceptibility against seven antibiotic classes using the Kirby-Bauer disk diffusion technique [Clinical Laboratory Standard Institute (CLSI), 2018]. The antibiotics included ampicillin (10 µg), amoxicillin-clavulanic acid/ augmentin (20 µg/10 µg), amoxicillin (10 µg), trimethoprim-sulfamethoxazole/ cotrimoxazole (1.25 µg/23.75 µg), cefoxitin (30 µg), cefepime (30 µg), imipenem (10 µg), neomycin (10 µg), tetracycline (30 µg), gentamycin (10 µg) and chloramphenicol (30 µg) (Mast Diagnostics, Randburg, SA) (CLSI, 2018). Break points measured were compared to those outlined by the CLSI (2018) for Enterobacteriaceae. Isolates resistant to

three or more antimicrobial classes were regarded as multidrug resistant. *E. coli* ATCC 25922 was included as a control (CLSI, 2018).

3.2.4 Molecular characterization of diarrheagenic *Escherichia coli*

The presence of different diarrheagenic *E. coli* virulence genes for ETEC (*lt* and *st* genes), EPEC (*bfpA* and *eaeA* genes), Eagg (*eagg* gene), EHEC (*eaeA*, *stx1* and *stx2* genes), and EIEC (*ipaH* gene) (Table 3.1) were analysed by PCR and sequencing, with the *mdh* gene used as internal control in all reactions. Control strains for the PCR reactions included DSM 10973 and DSM 27503 (ETEC); DSM 8703 and DSM 8710 (EPEC); DSM 27502 (Eagg); *E. coli* O157:H7 and ATCC 25922 (EHEC); and DSM 9028 and DSM 9034 (EIEC).

A single colony of each *E. coli* isolate was cultured aerobically under shaking conditions at 200 rpm in tryptone soy broth (TSB) (MERCK, Johannesburg) for 24 h at 30°C. The cells were pelleted by centrifugation (12,500 *g* for 10 min), DNA was extracted using the Quick-gDNA Mini-Prep kit (Zymo Research, Irvine, USA) and the DNA concentration was determined using the Qubit dsDNA Broad Range Assay and a Qubit 2.0 fluorometer (Life Technologies, Johannesburg). PCR was performed using the 1x DreamTaq Green PCR Master Mix (ThermoFisher Scientific, Johannesburg) with 60 – 100 ng DNA, with specific primers and thermocycling conditions for each of the genes (Table 3.1). The PCR products were visualized on a 2% agarose gel using a molecular imager (Gel Doc XR+, Bio-Rad).

Table 3.1: Primers used for screening of diarrheagenic *Escherichia coli* isolated from fresh produce sold formally and informally

Diarrheagenic *Escherichia coli*	Target genes	Primer sequences (5'-3')	Thermocycling conditions	Expected amplicon size (bp)	Reference
Enterotoxigenic (ETEC)	*lt*	F: GGC GAC AGA TTA TAC CGT GC R: CGG TCT CTA TAT TCC CTG TT	95°C, 15min; 35 cycles of 94°C, 45s; 55°C, 45s; 68°C, 2.5 min; 72°C 5 min	410	Omar and Barnard, 2010
	st	F: TTT CCC CTC TTT TAG TCA GTC AAC TG R: GGC AGG ATT ACA ACA AAG TTC ACA		160	Omar and Barnard, 2010
Enteropathogenic (EPEC)	*bfpA*	F: AAT GGT GCT TGC GCT TGC TGC R: GCC GCT TTA TCC AAC CTG GTA	94°C, 5min; 35 cycles of 94°C, 40s; 68°C, 60s; 72°C, 2min; 72°C 5 min	324	López-Saucedo et al., 2003
	eaeA	F: CTG AAC GGC GAT TAC GCG AA R: GAC GAT ACG ATC CAG	95°C, 15min; 35 cycles of (94°C, 45s; 55°C, 45s; 68°C; 2min	917	Omar and Barnard, 2010
Enteroaggregative (Eagg)	*eagg*	F: CTG GCG AAA GAC TGT ATC AT R: AAT GTA TAG AAA TCC GCT GTT	94°C, 5min; 35 cycles of 94°C, 40s; 57°C, 60s; 72°C, 2min; 72°C, 5 min	630	Aslani et al., 2011
	eagg	F: CTG GCG AAA GAC TGA ATC AT R: CAA TGT ATA GAA ATC CGC TGT T	94°C, 5min; 35 cycles of 94°C, 40s; 53°C, 60s; 72°C, 1min; 72°C, 5min	630	Aslani et al., 2011
Enterohemorrhagic (EHEC)	*eaeA*	F: CTG AAC GGC GAT TAC GCG AA R: GAC GAT ACG ATC CAG	95°C, 15min; 35 cycles of 94°C, 45s; 55°C, 45s; 68°C; 2min	917	Omar and Barnard, 2010
	stx1	F: ACA CTG GAT GAT CTC AGT GG R: CTG AAT CCC CCT CCA TTA TG	95°C, 15min; 35 cycles of 94°C, 45s; 55°C, 45s; 68°C; 2min	614	Omar and Barnard, 2010
	stx2	F: CCA TGA CAA CGG ACA GCA GTT R: CCT GTC AAC TGA GCA CTT TG		779	Omar and Barnard, 2010
Enteroinvasive (EIEC)	*ipaH*	F: GTT CCT TGA CCG CCT TTC CGA TAC CGT C R: GCC GGT CAG CCA CCC TCT GAG AGT AC	95°C 5min 35cycles of 95°C 60s; 60°C 90s; 72°C 2min 72°C 10 min	600	Aranda et al., 2004

3.2.5 Statistical analysis

Data were analyzed using SAS version 9.3 statistical software (SAS/STAT User's Guide, 1999). Analysis of variance was used to test for significant differences between group by product combinations. The Shapiro-Wilk test was performed on the standardized residuals to test for deviations from normality (Shapiro and Wilk, 1965). Student's protected t-LSD (Least significant difference) were calculated at a 5% significance level to compare means of significant source effects (Snedecor and Cochran, 1980).

3.3 Results

3.3.1 Microbiological analysis

Enumeration of coliforms, *E. coli* and Enterobacteriaceae showed similar ranges for the different vegetable types, regardless of the vendor groups where it was purchased (Figure 3.1). The coliforms enumerated from the different products across all vendor types in the current study ranged from 0.6-8.1 log CFU/g on spinach, 0.0-8.2 log CFU/g on tomatoes, 3.6-7.8 log CFU/g on lettuce, 0.0-6.5 log CFU/g on cucumber, and 0.7-6.8 log CFU/g on green bean samples (Figure 3.1; Appendix B Table B1). The mean coliform counts on spinach from the formal and informal markets were not significantly different, with the exception of the mean coliform counts on spinach from the trolley vendors (5.1 log CFU/g), which were significantly lower (p=0.0003) than that on spinach from the farmers' market vendors (6.0 log CFU/g) (Appendix B Table B1). Similarly, the coliform counts on tomatoes from the formal and informal markets were not significantly different, with the exception of the mean coliform count on tomatoes from trolley vendors (4.4 log CFU/g) being significantly lower (p=0.0003) than that on tomatoes from the farmers' market vendors (5.4 log CFU/g). Coliforms enumerated from cucumbers (4.1 log CFU/g) were significantly lower (p=0.0003) than the coliforms enumerated from the leafy green vegetables (spinach and lettuce).

Enterobacteriaceae enumerated from trolley vendor spinach samples (4.6 log CFU/g) were significantly lower (p=0.0082) than that of retailers (5.8 log CFU/g) and farmers' market vendors (5.9 log CFU/g) (Appendix B Table 1B). The Enterobacteriaceae counts on spinach ranged between 0.0-8.2 log CFU/g, on tomatoes between 0.0-8.1 log CFU/g, on lettuce between 4.2-8.3 log CFU/g, on cucumbers between 0.0-6.5 log CFU/g, and on green beans between 0.0-7.7 log CFU/g (Figure 3.1) (Appendix B Table B1).

Escherichia coli was enumerated from all the different produce types and sampling points, however not all samples were positive for *E. coli* after enrichment. Interestingly, the *E. coli* occurrence (number of samples positive for *E. coli* enumeration) were higher on tomatoes than spinach for all groups, except for produce from farmers' markets (Appendix B Table B1). Except for the farmers' market spinach that had mean *E. coli* counts of 1.2 log CFU/g, the *E. coli* counts on different produce types in the current study were < 10 CFU/g (Figure 3.1). The mean *E. coli* levels on spinach from the farmers' market vendors (1.2 log CFU/g) were significantly higher (p=0.0364) than that of spinach from street traders (0.3 log CFU/g). Overall, 90-98% of the tomato samples from the different vendors had satisfactory *E. coli* counts (100 – 1000 *E. coli* CFU/g), according to the commission regulation on microbiological criteria for ready-to-eat pre-cut fruit and vegetables (EC, 2007). Spinach samples from all the different vendors had satisfactory *E. coli* counts ranging from 70% of the spinach samples from farmers' market vendors to 94% of spinach samples from the street traders. Similarly, 82.0%, 93.3%, and 80.0% of the lettuce, cucumber, and green beans samples respectively, had satisfactory *E. coli* counts.

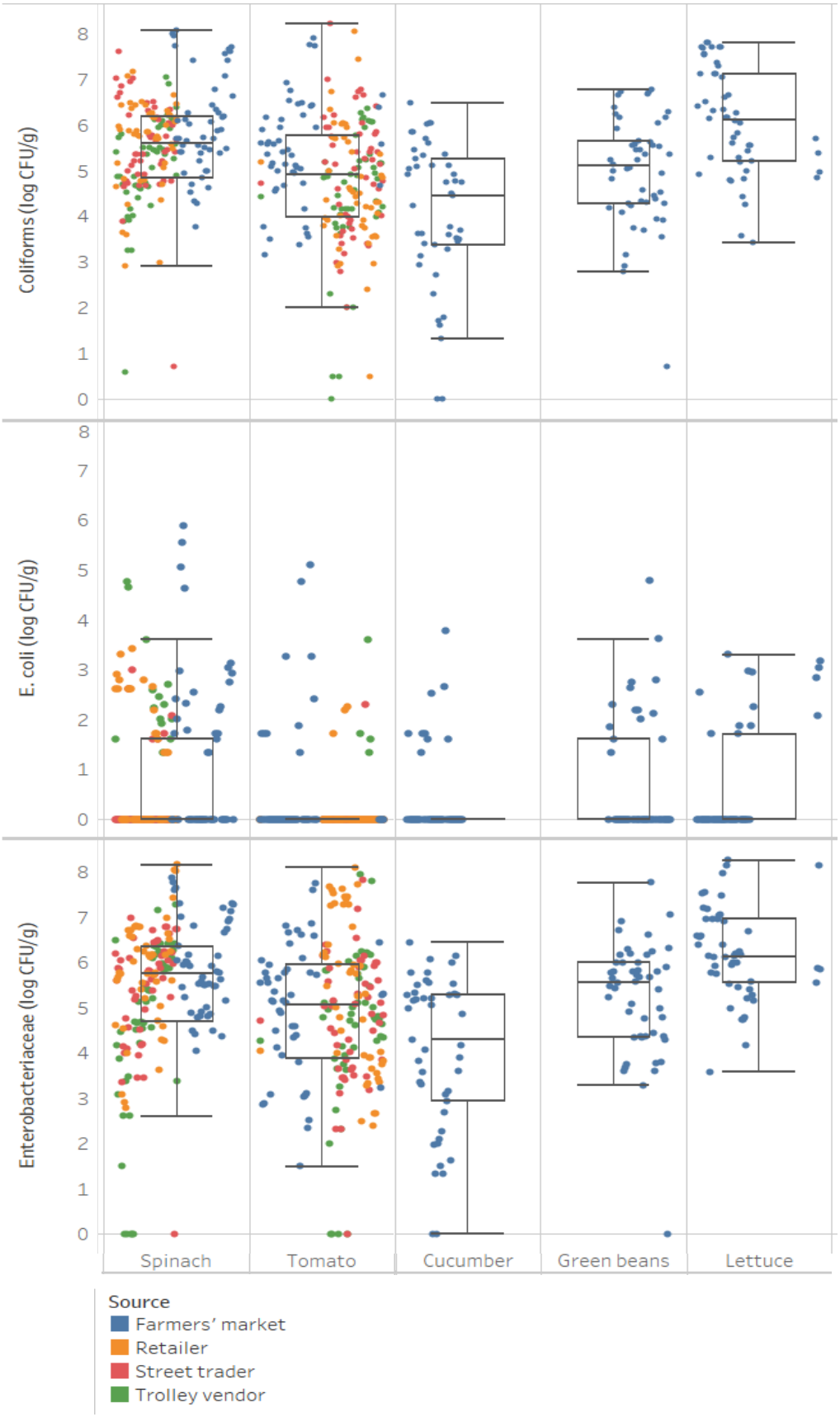

Figure 3.1: Coliform, *Escherichia coli* and Enterobacteriaceae counts (log CFU/g) on spinach, tomato, cucumber, green bean and lettuce samples purchased from formal and informal markets in Gauteng Province, South Africa.

3.3.2 Detection of potential foodborne pathogens

In the current study, 14.86% (81/545) of the vegetable samples analysed from all the different vendor types harboured *E. coli* after enrichment. This included 62/245 (25.30%) farmers' market samples, 6/100 (6.00%) street traders' samples, 3/100 (3.00%) trolley vendor samples, and 10/100 (10.00%) samples from retailers. The highest occurrence of *E. coli* isolates following enrichment was from the leafy green vegetable samples; 15/50 (30.00%) farmers' market spinach samples, 7/50 (14.00%) farmers' market lettuce samples, 4/50 (8.00%) street traders' spinach samples, 3/50 (6.00%) trolley vendor spinach samples and 8/50 (16.00%) retailers' spinach samples. *Escherichia coli* from tomatoes in the current study were isolated from 14.00% (7/50) of the farmers' market tomato samples and 2/50 (4.00%) street trader- and retailer tomato samples, respectively. From the farmers' market green bean samples (n=50), 13 samples (26.00%) were contaminated with *E. coli*, whilst 9/45 (20.00%) of the farmers' market cucumber samples were contaminated with *E. coli*. No *Salmonella* spp. nor *Listeria* spp. were detected on any of the samples from any of the different vendors. From the 67 selected *E. coli* isolates for further characterisation, none were positive for any of the diarrheagenic virulence genes.

3.3.3 Phenotypic antimicrobial resistance profiling of *Escherichia coli* isolates

From the 67 selected *E. coli* isolates, resistance were observed against all the antibiotics screened for, with resistance against neomycin the highest (73.13%) followed by penicillins (ampicillin, 38.81% and amoxycillin, 41.79%), sulfonamides (cotrimoxazole, 22.39%), tetracycline (19.40%) and chloramphenicol (11.94%) (Figure 3.2). Less than 10% of the isolates were resistant to cefoxitin, imipenem, and gentamycin, respectively. Overall, multidrug resistance (resistance to ≥3 antibiotic classes) was observed in 40.30% of the *E. coli* isolates. The most frequent resistance patterns within the different antibiotic classes for the isolates included resistance to antibiotics in the Penicillins-Cephalosporins-Aminoglycosides

combination (13 MDR isolates), followed by the Penicillins-Aminoglycosides-Sulfonamides-Tetracyclines-Chloramphenicol combination (five isolates) and the Penicillins-Cephalosporins-Aminoglycosides-Sulfonamides (three isolates) combination (Appendix B Table B2).

Chapter 3

Figure 3.2: Phenotypic antimicrobial resistance profiles of *E. coli* isolated from different fresh produce types sold at different vendors in Gauteng Province, South Africa.

3.4 Discussion

This study is the first to investigate the microbiological quality (including Enterobacteriaceae enumeration) and occurrence of multidrug resistant (MDR) generic *E. coli* in comparing fresh vegetables sold at retailers, street vendors, trolley vendors and farmers' markets in Gauteng Province. The microbiological quality of fresh produce, mainly leafy greens, sold at different markets have been studied worldwide (Korir et al., 2016; du Plessis et al., 2017; Quansah et al., 2018; Roth et al., 2018). Leafy greens have previously been prioritized as the highest level of concern in terms of fresh produce safety from a global perspective (WHO, 2008). The WHO has further stated that produce of second highest concern (level 2 priority) include tomatoes and green onions, whilst carrots and cucumbers amongst others were a level 3 priority.

The fresh produce samples from retailers, street traders, trolley vendors and farmers' markets collectively had a high prevalence of coliforms (≥ 90%), compared to the 52.0-75.6% coliform prevalence on vegetables from retailers and farmers' markets in Florida, U.S. (Roth et al., 2018), and 38.7% prevalence on vegetables from retail stores on the eastern shore of Maryland, USA (Korir et al., 2016). Regardless of the vegetable type, Roth et al. (2018) found produce from retailers to have constant lower coliform prevalence than the farmers' market vegetables. In contrast, the results from the current study were similar to a previous South African study where 100% of spinach samples from retailers as well as from street vendors were positive for coliforms (du Plessis et al., 2017), with no significant difference in coliform counts observed in the vegetables from formal and informal markets. The guidelines with regard to acceptable hygiene indicator bacteria counts on RTE produce differ across the world (FSANZ, 2001; Health Protection Agency, 2009; FSAI, 2016). Moreover, the SA Department of Health's microbiological guidelines for fresh fruits and vegetables to be eaten raw are currently being revised. Other countries do not include coliform counts in the guidelines for interpretation of

results of microbiological testing of RTE foods, which should be considered in the revision process of the SA guidelines.

Naturally, coliform and Enterobacteriaceae counts of vegetables are often > 4 log CFU/g. Enterobacteriaceae as indicators within fresh produce safety is therefore often excluded, due to the natural occurrence and complex relationship between indicator microorganisms such as coliforms and foodborne pathogens (FAO and WHO, 2019). Coliforms include amongst other *Citrobacter*, *Klebsiella*, *Enterobacter* and *E. coli*, that could potentially pose a threat to human health (Baylis et al., 2011). Yet, as the coliform bacteria fall within the greater Enterobacteriaceae family, the significance of a high prevalence on vegetables is understandable and must be put into context due to the natural association with plants (Baylis et al., 2011). The overall Enterobacteriaceae loads observed on the different vegetable types in the current study corresponded to results previously reported (Abadias et al., 2008; Al-Holy et al., 2013; Al-kharousi et al., 2016). The Enterobacteriaceae counts on different vegetables from formal and informal markets reiterated the natural bacterial prevalence on the produce, regardless of food safety regulations being implemented or not in these contrasting points of sale with highly differing personal hygiene and sanitation standards and cold refrigeration capacity (Al-kharousi et al., 2016; Grace et al., 2019).

In the current study, *E. coli* was enumerated from all the different produce types and sampling points, however not all samples were positive for *E. coli* after enrichment. Except for the farmers' market spinach that had mean *E. coli* counts of 1.2 log CFU/g, the *E. coli* counts on different produce types in the current study were < 10 CFU/g. This is similar to previous *E. coli* levels reported on spinach and cabbage from retailers and street vendors in SA (Du Plessis et al., 2017), and lower than *E. coli* counts on spinach from retailers (1.0 -1.8 log CFU/g) in the United States (U.S.) (Korir et al., 2018). Although the majority of *E. coli* counts on fresh produce was acceptable, some samples was of poor microbiological quality, which corresponds

to previous reports of potential foodborne pathogen contamination in fresh produce in developing countries (Mir et al., 2018). Overall, 2-8% of the tomato samples from the different vendors had unsatisfactory *E. coli* counts (*E. coli* ≥ 1000 CFU/g), according to the commission regulation on microbiological criteria for RTE pre-cut fruit and vegetables (European Commission [EC], 2007). Spinach samples from all different vendors had unsatisfactory *E. coli* counts ranging between 12% from farmers' market vendors to 6%, 4%, and 2% from trolley vendors, retailers and street traders respectively. Similarly, 6%, 4%, and 2% lettuce, green beans, and cucumber samples respectively, had unsatisfactory *E. coli* counts. When evaluated against international guidelines as specified in the United Kingdom (UK) (20 to 100 CFU/g), Australia (3 to 100 CFU/g), and Canada (100 most probable number per g), 13.03% (n=71) of the samples from the current study would not have been compliant (FSANZ, 2001; Health Protection Agency, 2009; Health Canada, 2010). This included 19.72% (n=14) samples from the formal- and 80.28% (n=57) samples from the informal markets, respectively. The high percentage (50%) of the SA population that depend on informal trade, highlights the need to improve fresh produce safety in all the different markets (Petersen and Charman, 2018). In SA, 21.76% and 95.60% of the population purchasing from the informal sector consume raw and/or cooked spinach and tomatoes, respectively. The questionnaire survey results from the population purchasing from the formal sector, showed that 94%, 29% and 94% of the respondents eat lettuce, beans and cucumber raw, respectively (Water Research Commission [WRC], 2018; Baloyi, 2020).

In contrast to Du Plessis et al. (2017), no *Salmonella* spp. nor *L. monocytogenes* were detected from any of the vegetables in the current study after PCR confirmation. In the current study, 14.86% (81/545) of the vegetable samples analysed from all the different vendor types harboured *E. coli* after enrichment. Previously, Du Plessis et al. (2017) reported *E. coli* presence in 0-73% of spinach samples from formal retailers and in 50-100% of street vendor

spinach samples, all purchased in Johannesburg. This occurrence was higher than the *E. coli* isolated from 16% formal retailers, 8% street traders, and 6% trolley vendor spinach samples in this study. Furthermore, Scheinberg et al. (2017) reported that 29.00% and 17.00% of lettuce and spinach samples respectively, were positive for generic *E. coli* from farmers' markets in Pennsylvania, whilst in the current study, 14.00% and 30.00% of the farmers' market lettuce and spinach samples respectively, were positive for generic *E. coli.* In contrast to other studies that have reported on spinach and lettuce contaminated with *E. coli* harbouring *stx2* and *eae* genes (Li et al., 2016) and *E. coli* isolates characterised as EAEC, EPEC and ETEC positive strains (Waturangi et al., 2019), none of the 67 selected *E. coli* isolates for further characterisation from the current study harboured virulence genes. The presence of *E. coli* on fresh produce however remains significant, as these potential pathogens can be an additional reservoir of antimicrobial resistance genes (Luna-Guevara et al., 2019).

Antimicrobial resistance genes can readily be transferred to commensal bacteria, including non-pathogenic bacteria, that typically colonise the human gut and are therefore regarded as emerging environmental contaminants (du Plessis et al., 2017). The natural occurrence of Enterobacteriaceae and higher microbial loads of potential pathogens such as *E. coli* therefore becomes concerning when investigating the possibility of fresh produce aiding in dissemination of clinically important resistance genes (Vikesland et al., 2017). Overall, multidrug resistance (resistance to ≥3 antibiotic classes) was observed in 40.30% of the *E. coli* isolates. This was similar to the 37.90% multidrug-resistance reported in *E. coli* isolates from spinach in another SA study (Du Plessis et al., 2017), but lower than the 100% multidrug resistance reported in *E. coli* from lettuce and cabbage in Ghana (Adzitey, 2018). Except for one cucumber *E. coli* isolate, the *E. coli* isolates from all product types were, similar to results reported by Du Plessis et al. (2017), susceptible to second generation cephalosporin antibiotics (cefoxitin). In addition, 34.30% of the isolates were resistant to fourth-generation

cephalosporin antibiotics (cefepime) and < 10% resistant to impenem (carbapenemase). Environmental *E. coli* with multidrug-resistance phenotypes have similarly been described in previous reports, including in developing countries (Canizalez-Roman et al., 2019; Corzo-Ariyama et al., 2019; Du Plessis et al., 2017). With a rise in antimicrobial resistance in both commensal and pathogenic bacteria in different environments, subsequent treatment options to infections become limited (Freitag et al., 2018).

3.5 Conclusion

This study showed that *E. coli* levels on spinach and tomatoes from the retailers, street traders, trolley vendors and farmers' markets were not significantly different. Furthermore, the farmers' market lettuce samples also showed similar *E. coli* levels to the spinach from all the different groups tested. No *Salmonella* spp. or *L. monocytogenes* were detected nor isolated from any of the vegetables sampled in this study. However, the prevalence of multidrug-resistant commensal *E. coli* highlights the need for improved food safety practices within the supply chains and identification of sources of fresh produce contamination with antimicrobial resistant bacteria as a public health concern. The antimicrobial resistance levels observed in commensal *E. coli* isolated from fresh produce at the point of sale further highlights the need to include characterisation of Enterobacteriaceae (commensal and potential pathogenic bacteria) with expanded spectrum antimicrobial resistance, as well as surveillance of fresh produce production systems from farm-to-retail, to identify potential sources of contamination which contribute to the presence and dissemination of antimicrobial resistant microorganisms and their genetic determinants.

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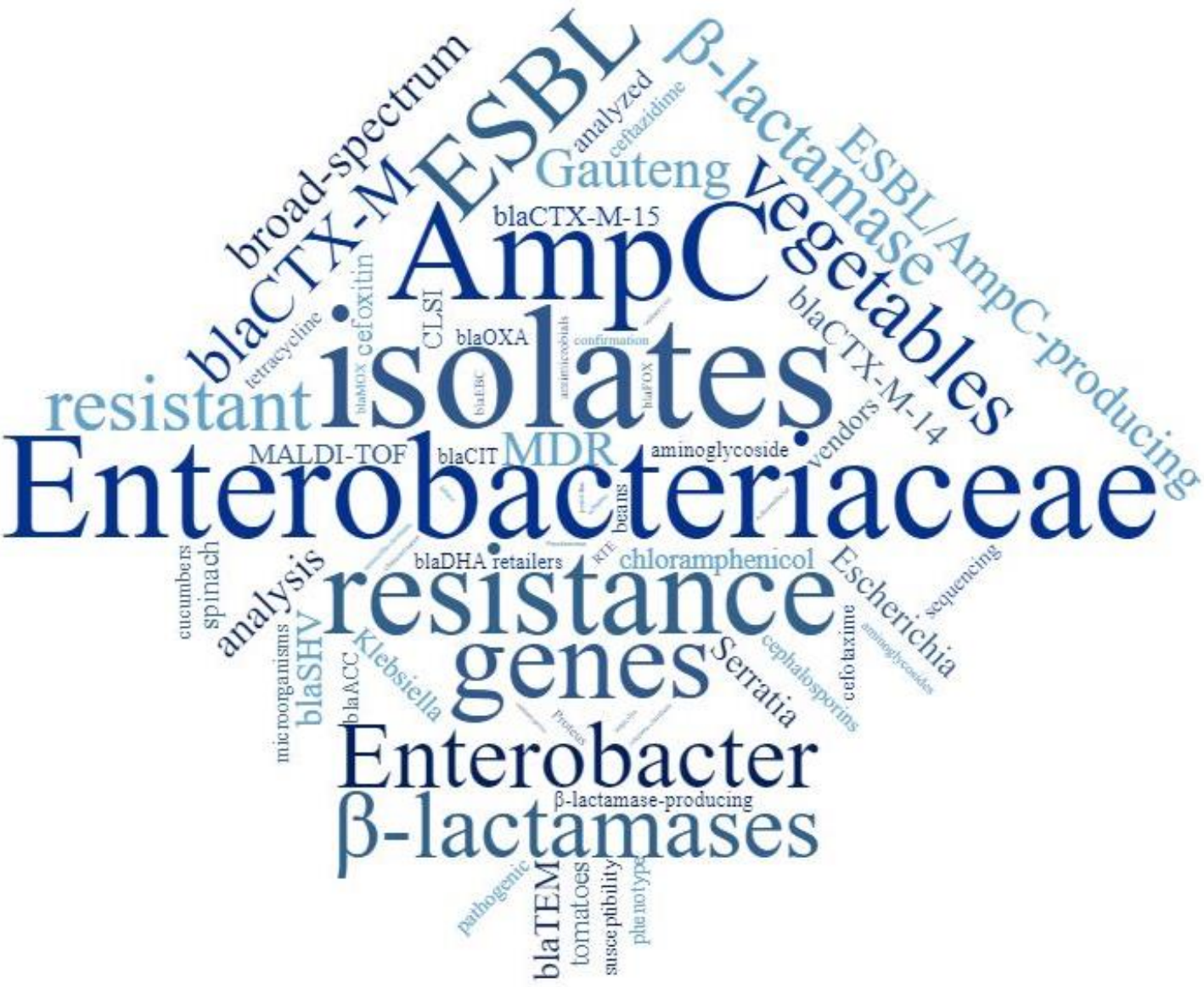
Chapter 4

"Lots of people think, well, we're humans; we're the most intelligent and accomplished species; we're in charge. Bacteria may have a different outlook: more bacteria live and work in one linear centimeter of your lower colon than all the humans who have ever lived. That's what's going on in your digestive tract right now.
Are we in charge, or are we simply hosts for bacteria?
It all depends on your outlook." -*Neil deGrasse Tyson*



Fresh Produce at the Point of Sale

10 Retailers | 10 Trolley vendors | 10 Street traders | 13 Farmers' market vendors

545 Samples

Characterisation of **E**xtended-**S**pectrum and AmpC **β**-**L**actamase-producing Enterobacteriaceae

17.4% (95/545) vegetable samples were contaminated with ESBL/AmpC-producing Enterobacteriaceae

Dominant species identified from the 10 genera isolated:

- *Escherichia coli*
- *Enterobacter cloacae*
- *Enterobacter asburiae*
- *Klebsiella pneumoniae*

77 Characterised isolates:

- 96.1% Multidrug resistant
- Most prevalent resistance:
  aminoglycoside (94.8%)
  chloramphenicol (85.7%)
  tetracycline (53.2 %)
- ESBL production in 79.2% isolates
- AmpC production in 41.6% isolates

Neccesity of surveillance of fresh produce production systems from farm to retail and identification of potential sources of contamination which contribute to the presence and dissemination of antimicrobial resistant microorganisms

First report of multidrug resistant ESBL/AmpC producing Enterobacteriaceae in raw vegetables sold at selected formal and informal markets in Gauteng Province, South Africa.

Richter, L., du Plessis, E.M., Duvenage, S., and Korsten, L.(2019). Occurrence, Identification, and Antimicrobial Resistance Profiles of Extended-Spectrum and AmpC β-Lactamase-Producing Enterobacteriaceae from Fresh Vegetables Retailed in Gauteng Province, South Africa. Foodborne Pathog. Dis. 16, 421–427. doi:10.1089/fpd.2018.2558.

Plant to Food

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Occurrence, identification and antimicrobial resistance profiles of extended-spectrum and AmpC β-lactamase-producing Enterobacteriaceae from fresh vegetables retailed in Gauteng Province, South Africa[3]

Abstract

Extended-spectrum β-lactamase (ESBL) and AmpC β-lactamase-producing Enterobacteriaceae are no longer restricted to the health care system, but represent increased risks related to environmental integrity and food safety. Fresh produce has been increasingly reported to constitute a reservoir of multidrug resistant potential human pathogenic Enterobacteriaceae. This study aimed to detect, identify and characterize the antimicrobial resistance of ESBL/AmpC-producing Enterobacteriaceae isolates from fresh vegetables at point-of-sale. Vegetable samples [spinach, tomatoes, lettuce, cucumber and green beans (n=545)] were purchased from retailers in Gauteng, the most densely populated province in South Africa. These included street vendors, trolley vendors, farmers' market stalls and supermarket chain stores. Selective enrichment, plating onto chromogenic media and matrix-assisted laser desorption ionization time-of flight mass spectrometry (MALDI-TOF MS) confirmation of isolate identities, showed that 17.4% (95/545) vegetable samples analyzed were contaminated with presumptive ESBL/AmpC-producing Enterobacteriaceae. Dominant species identified included *Escherichia coli, Enterobacter cloacae, Enterobacter asburiae and Klebsiella pneumoniae.* Phenotypic antibiotic resistance analysis showed that 96.1% of 77 selected isolates were multidrug resistant, while resistance to aminoglycosides (94.8%), chloramphenicol (85.7%) and tetracyclines (53.2 %) antibiotic classes were most prevalent.

[3] **Published as:** Richter, L., du Plessis, E.M., Duvenage, S., and Korsten, L. **(2019).** Occurrence, Identification, and Antimicrobial Resistance Profiles of Extended-Spectrum and AmpC β-Lactamase-Producing Enterobacteriaceae from Fresh Vegetables Retailed in Gauteng Province, South Africa. *Foodborne Pathog. Dis.* 16, 421–427. doi:10.1089/fpd.2018.2558.

Positive phenotypic analysis for ESBL production were shown in 61 (79.2%) of the 77 isolates and AmpC production in 41.6% of the isolates. PCR and sequencing confirmed the presence of β-lactamase genes in 75.3% isolates from all vegetable types analyzed, mainly in *E. coli*, *Enterobacter* spp. and *Serratia* spp. isolates. CTX-M group 9 (32.8%) was the dominant ESBL type, while EBC (24.1%) was the most prevalent plasmidic type AmpC β-lactamase. Our findings document, for the first time, the presence of multidrug resistant ESBL/AmpC producing Enterobacteriaceae in raw vegetables sold at selected retailers in Gauteng Province, South Africa.

4.1 Introduction

Extended-spectrum β-lactamase (ESBL)- and AmpC-producing Enterobacteriaceae have increased in occurrence globally in health care systems, agroecosystems and fresh produce, due to the widespread use of broad-spectrum antibiotics (Ye et al., 2017a). Dissemination of these antimicrobial resistant microorganisms have been identified as one of the six main antibiotic resistance (AMR) related health risks globally (WHO, 2015). If infection by ESBL/AmpC-producing Enterobacteriaceae occurs, treatment options become limited as a result of expanded AR of the corresponding isolates (Freitag et al., 2018). Since ESBL/AmpC β-lactamases are capable of inactivating broad spectrum penicillins and cephalosporins, their presence in Enterobacteriaceae are of clinical and epidemiological importance (Kolar et al., 2010). Clinically important ESBL-producing Enterobacteriaceae have been reported in different South African provinces [Eastern Cape (Vasaikar et al., 2017); Western Cape (Peirano et al., 2011); KwaZulu-Natal (Mahomed and Coovadia, 2014); and Gauteng (Ehlers et al., 2009). In 53 clinical isolates from Gauteng, ESBL gene prevalence was reported in 87 % (Ehlers et al., 2009).

ESBLs, classified as Ambler Class A enzymes, include TEM-, SHV- and CTX-M-type enzymes (Östholm, 2014; Ghafourian et al., 2015). More than 200 TEM and SHV variants have been documented, while 90 different enzymes within the CTX-M type have been described (Östholm, 2014; Bush and Bradford, 2019)). Class A enzymes hydrolyse ampicillin and extended-spectrum cephalosporins (Ghafourian et al., 2015; Bush and Bradford, 2019)). Bacteria expressing AmpC β-lactamases, classified as Class C enzymes, are resistant to additional β-lactams, i.e. cephamycins, and are not influenced negatively by class A enzyme inhibitors (Jacoby, 2009; Njage and Buys, 2017). Plasmid-mediated AmpC (pAmpC)-producing strains are distinguished from chromosomal AmpC since they are often not inducible (Mezzatesta et al., 2012). Six families of pAmpC-β-lactamases including CIT, FOX, MOX, DHA, EBC and ACC have been described, with DHA, CMY (CIT family member) and FOX most commonly detected (Thomson, 2010). Co-occurrence of β-lactamase enzymes, especially AmpC β-lactamases and ESBLs, are common (Thomson, 2010).

Salmonella spp., pathogenic *Escherichia coli* and *Shigella* spp. have been implicated in foodborne disease (FBD) outbreaks, while *Klebsiella pneumoniae*, *Serratia marcescens*, *Citrobacter freundii*, and *Enterobacter* spp. are regarded as opportunistic human pathogenic bacteria (Baylis et al., 2011). The presence of ESBL/AmpC-producing Enterobacteriaceae on fresh produce has been studied worldwide (Kim et al., 2015; Nüesch-Inderbinen et al., 2015; Zurfluh et al., 2015). Transfer of multidrug resistant (MDR) Enterobacteriaceae onto fresh produce occur through the use of contaminated irrigation water or during production via animal manure (van Hoek et al., 2015). Subsequent transfer to humans can happen through consumption of raw vegetables, potentially impacting consumer health negatively (Ye et al., 2017a). Concomittantly AMR genes can easily be transferred to commensal bacteria which typically colonize the human gut.

Fresh vegetables produced in South Africa (SA) are retailed nationally and to the South African Development Community (SADC) countries, Swaziland, the United Kingdom (UK), Middle East and Asian markets (DAFF, 2012a, 2012b, 2016). Current knowledge regarding the occurrence of ESBL/AmpC- producing Enterobacteriaceae on fresh vegetables in SA is limited. The aim of this exploratory study was to detect, to identify and to characterize the AR of ESBL- and AmpC-producing Enterobacteriaceae isolates from frequently consumed fresh vegetables from selected retailing sites in Gauteng Province.

4.2 Materials and Methods

4.2.1 Sample collection

As described in Chapter 3, a total number of 545 vegetable samples was collected from 10 formal retailers, 10 street trading greengrocers, 10 mobile trolley vendors, and 13 vendors at two farmers' markets in Gauteng Province, from September 2017 to May 2018. In the informal markets, street traders typically display fresh produce on a table, underneath a shade covering, at the roadside or they use mobile trolleys. The vegetable samples included, depending on availability, spinach (bunches, baby leaves, or minimally processed ready-to-eat (RTE) pillow packs) (n=200), tomatoes (n=200), cucumbers (n=45), lettuce (Iceberg lettuce heads or mixed salad leaf RTE pillow packs) (n=50), and green beans (n=50 samples). All samples were transported in cooler boxes and stored at 4 °C until further processing within 24 h.

4.2.2 Processing of fresh produce

The fresh produce samples were processed as described in Chapter 3. Briefly, at least three leaves from one spinach bunch and the inner leaves of three lettuce heads were used to prepare 50 g composite samples of each of the leafy vegetable samples. Each spinach or lettuce sample were aseptically cut into a sterile polyethylene strainer stomacher bag containing 200 ml buffered peptone water (BPW) (3M, Johannesburg, SA) in a 1:4 weight to volume ratio. A 150

g sample of tomatoes and cucumbers (composite of at least three tomatoes or cucumbers) and a 150 g sample of green beans were each placed into a sterile polyethylene stomacher bag containing 150 ml BPW in a 1:1 weight to volume ratio (Xu et al., 2015). Individual vegetable samples were blended for 5 min at 230 rpm in a Stomacher 400 circulator paddle blender (Seward Ltd., London, UK).

4.2.3 Isolation and identification of presumptive extended-spectrum and AmpC β-lactamase- producing Enterobacteriaceae

Each of the BPW-sample mixtures was incubated for 3-4 h at 37 °C after which 1 ml of each sample was added to 9 ml Enterobacteriaceae enrichment (EE) broth (Oxoid, Johannesburg) according to ISO 21528-1:2004 and incubated overnight at 30 °C (Blaak et al., 2014c). ESBL-producing microorganisms were detected by streaking 10 µl of each of the enriched samples onto ChromID ESBL agar plates (bioMérieux, Midrand, SA) and incubated overnight at 30 °C (Blaak et al., 2014c). All presumptive positive ESBL/AmpC- producing Enterobacteriaceae colonies based on colony colour, including weakly coloured colonies, on the chromogenic media were isolated and purified. Isolate identities were determined using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF) (Bruker, Bremen, Germany) to species level as described by Standing et al. (2013). A single colony on nutrient agar were transferred to the MALDI-TOF polished steel target plate and further analysed according to manufacturer's instructions (AOAC-OMA#2017.09), following calibration with the bacterial test standard (Appendix C, Table C1). Non-Enterobacteriaceae isolates were not included in further analysis.

4.2.4 Antimicrobial susceptibility testing

A selection of 77 presumptive ESBL producing Enterobacteriaceae isolates, representing all unique species per product type from each supplier, were selected for further analysis. The Kirby-Bauer disk diffusion technique was used to determine the resistance patterns of the

isolates [Clinical Laboratory Standard Institute (CLSI, 2018)]. All isolates were screened for ESBL production by the double-disk synergy test (DDST) using cefotaxime-30 µg, ceftazidime-30 µg, and cefpodoxime-10 µg, alone or in combination with clavulanic acid-10 µg (Mast Diagnostics, Randburg) (EUCAST, 2013). Zone diameters were compared to the CLSI and EUCAST criteria to determine if isolates were resistant, intermediate or susceptible. Isolates showing resistance to cefoxitin and cefotaxime or ceftazidime were regarded as a phenotypic indicator of AmpC production (EUCAST, 2013). Production of ESBLs were confirmed using the cefepime ESBL disc set (Cefepime-30 µg, cefepime-clavulanic acid-30 µg-10 µg) and AmpC production using the AmpC detection set (Mast Diagnostics, Randburg) (EUCAST, 2013; CLSI, 2018). Additional antimicrobials tested for resistance or susceptibility of isolates included ampicillin-10 µg, amoxicillin-clavulanic acid-20 µg/10 µg, amoxicillin-10 µg, trimethoprim-sulfamethoxazole-1.25µg/23.75 µg, imipenem-10 µg, neomycin-10 µg, tetracycline-30 µg, gentamycin-10 µg, chloramphenicol-10 µg (Mast Diagnostics, Randburg, SA) (CLSI, 2018). Isolates resistant to three or more antimicrobial classes were regarded as MDR. *Klebsiella pneumoniae* ATCC 700603, *Escherichia coli* NCTC 13315, *Enterobacter cloacae* NCTC 1406, and *Escherichia coli* ATCC 25922 were included as positive and negative controls as described by the manufacturer (Mast Diagnostics).

4.2.5 Characterization of β-lactamase genes

The presence of ESBL determinants (bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, bla_{OXA}) and pAmpC resistance genes (bla_{ACC}, bla_{FOX}, bla_{MOX}, bla_{DHA}, bla_{CIT}, bla_{EBC}) in the selected isolates were analysed by PCR and sequencing. Single colonies of each presumptive ESBL-producing Enterobacteriaceae isolate were cultured aerobically under shaking conditions at 200 rpm in tryptone soy broth (MERCK, Johannesburg) for 24 h at 30 °C. The cells were pelleted by centrifugation (12,500 *g* for 10 min), DNA was extracted using the Quick-gDNA Mini-Prep

kit (Zymo Research, Irvine, USA) and the DNA concentration was determined using the Qubit dsDNA Broad Range Assay and a Qubit 2.0 fluorometer (Life Technologies, Johannesburg). PCR was performed using the DreamTaq Green PCR Master Mix (ThermoFisher Scientific, Johannesburg), specific primers, and thermocycling conditions for each of the genes as described in Table 4.1. PCR products were sequenced using BigDye Terminator v3.1 cycle sequencing on an ABI 3500XL sequencer in forward and reverse direction (InquabaBiotec, Johannesburg). The sequences were edited with Chromas 2.6 and BioEdit sequence alignment editor software and consensus sequences were subjected to BLAST nucleotide search analysis to identify the AMR genes.

Table 4.1: Primers used for screening of broad-spectrum β-lactamase, ESBL and AmpC genetic determinants in selected Enterobacteriaceae isolates from fresh produce samples (Dallenne et al., 2010)

Target genes	Primer sequences	Thermocycling conditions	Expected amplicon size (bp)
bla$_{TEM}$	TEM-F: 5'-CATTTCCGTGTCGCCCTTATTC-3' TEM-R: 5'-CGTTCATCCATAGTTGCCTGAC-3'	94°C, 10min; 30 cycles of 94°C, 40s, 58°C, 40s, 72°C 1min; 72°C 7min	800
bla$_{SHV}$	SHV-F: 5'-AGCCGCTTGAGCAAATTAAAC-3' SHV-R: 5'-ATCCCGCAGATAAATCACCAC-3'		713
bla$_{OXA-1\ like}$	OXA-F: 5'-GGCACCAGATTCAACTTCAAG-3' OXA-R: 5'-GACCCCAAGTTTCCTGTAAGTG-3'		564
bla$_{CTX-M\ Group\ 8/25}$	CTX-M Gp8/25-F: 5'-AACRCRCAGACGCTCTAC-3' CTX-M Gp8/25-R: 5'-TCGAGCCGGAASGTGTYAT-3'	94°C, 10min; 30 cycles of 94°C, 40s, 60°C, 40s, 72°C 1min; 72°C 7min	326
bla$_{CTX-M\ Group\ 9}$	CTX-M Gp9-F: 5'-TCAAGCCTGCCGATCTGGT CTX-M Gp9-R: 5'-TGATTCTCGCCGCTGAAG-3'		688
bla$_{CTX-M\ Group\ 1}$	CTX-M Gp1-F: 5'-TTAGGAARTGTGCCGCTGYA-3' CTX-M Gp1-R: 5'-CGATATCGTTGGTGGTRCCAT-3'		561
bla$_{ACC}$	ACC-F: 5'-CACCTCCAGCGACTTGTTAC-3' ACC-R: 5'-GTTAGCCAGCATCACGATCC-3'	94°C, 10min; 30 cycles of 94°C, 40s, 60.5°C, 40s, 72°C 1min; 72°C 7min	346
bla$_{FOX}$	FOX-F: 5'-CTACAGTGCGGGTGGTTT-3' FOX-R: 5'-CTATTTGCGGCCAGGTGA-3'	94°C, 10min; 30 cycles of 94°C, 40s, 59.6°C, 40s, 72°C 1min; 72°C 7min	162
bla$_{MOX}$	MOX-F: 5'-GCAACAACGACAATCCATCCT-3' MOX-R: 5'-GGGATAGGCGTAACTCTCCCAA-3'		895
bla$_{DHA}$	DHA-F: 5'-TGATGGCACAGCAGGATATTC-3' DHA-R: 5'-GCTTTGACTCTTTCGGTATTCG-3'		997
bla$_{CIT}$	CIT-F: 5'-CGAAGAGGCAATGACCAGAC-3' CIT-R: 5'-ACGGACAGGGTTAGGATAGY-3'		538
bla$_{EBC}$	EBC-F: 5'-CGGTAAAGCCGATGTTGCG-3' EBC-R: 5'-AGCCTAACCCCTGATACA-3'		683

4.3 Results

4.3.1 Identification of presumptive extended-spectrum and AmpC β-lactamase-producing Enterobacteriaceae isolates

Using MALDI-TOF analysis, 122 (28.2 %) of the 432 presumptive extended-spectrum/AmpC β-lactamase-producing isolates obtained from the fresh vegetable samples were confirmed as Enterobacteriaceae belonging to ten genera. The 310 non-Enterobacteriaceae isolates were predominantly identified as *Pseudomonas* spp. The Enterobacteriaceae[4] isolates were identified as *Enterobacter* spp. (28.7 %), including *E. cloacae*, *E. asburiae*, *E. cowanii*, and *E. ludwigii*; *Serratia* (18.9 %), including predominantly *S. fonticola*; *Escherichia coli* (18 %); *Klebsiella* spp. (14.8 %), including *K. pneumoniae* and *K. oxytoca*; *Rahnella aquatilis* (9 %); *Proteus* spp. (4.9 %), including *P. penneri* and *P. mirabilis*; *Citrobacter* spp. (2.5 %), including *C. farmeri* and *C. freundii*; *Kluyvera ascorbata* (1.6 %); *Achromobacter xylosixidans* (1.6 %) and *Raoultella ornithinolytica* (0.8 %). Presumptive ESBL/AmpC-producing Enterobacteriaceae were isolated from the vegetable types tested.

4.3.2 Phenotypic antibiotic resistance profiling

All the 77 selected presumptive ESBL-producing Enterobacteriaceae showed resistance to more than one antimicrobial agent, with 96.1 % being MDR (resistant to ≥ 3 antimicrobial classes) (Figure 4.1). Resistance to the aminoglycoside and chloramphenicol classes were dominant, observed in 94.8 % and 85.7 % of the isolates respectively. All isolates with cephalosporin resistance (CTX30C, CAZ30C, CPD10C or CPM30C) were further screened using DDST, after which 61/77 (79.2 %) were tested positive for ESBL production (Figure

[4] A taxonomy change was adopted in 2020 to use “Enterobacterales” as the name of a new scientific order. "Enterobacteriaceae” are now one of seven families within the order, with certain members such as Serratia spp. now members of the family Yersiniaceae and Providencia spp. and Morganella spp. are members of the family Morganellaceae. This thesis however presents the data according to the previous classification where the order “Enterobacteriales” had a single Enterobacteriaceae family.

4.1). All isolates that showed cefoxitin resistance (n=46), were additionally screened with the AmpC detection set. From these 46 isolates, 32/77 (41.6 %) tested positive for AmpC production. This included 27 isolates showing resistance to cefoxitin, ceftazidime and/or cefotaxime and additionally five isolates that showed cefoxitin resistance, but ceftazidime and/or cefotaxime susceptibility. All isolates displaying ESBL or AmpC phenotypes were further characterized for identification of ESBL and/or AmpC resistance genes.

4.3.3 Genotypic antibiotic resistance profiling

Genes encoding β-lactamases were detected in 58/77 (75.3 %) isolates obtained from all vegetable types, mainly in *E. coli* (n=20), *Enterobacter* spp. (n=12), and *Serratia* spp. (n=11) isolates. This included 37 (48 %) broad-spectrum, 39 (51 %) ESBL and 20 (25.9 %) AmpC genetic determinants (Figure 4.1). The most frequently detected β-lactamase genes were $bla_{\text{CTX-M}}$ (n=28), followed by bla_{SHV} (n=22), bla_{TEM} (n=21) and bla_{OXA} (n=5). Extended-spectrum β-lactamases encoded by $bla_{\text{CTX-M}}$ included CTX-M-14 (n=15), CTX-M-15 (n=6), CTX-M-27 (n=4), and CTX-M-55 (n=3); bla_{TEM} genes encoded TEM-3 (n=3), while bla_{SHV} genes encoded SHV-18 (n=6), SHV-28 (n=1) and SHV-154 (n=1). All the bla_{OXA}, 85.7 % (n=18) of the bla_{TEM}, and 63.6 % (n=14) of the bla_{SHV} sequences encoded broad-spectrum β-lactamases OXA-1, TEM-1, TEM-215, SHV-1, SHV-11, or SHV-26 respectively. Three isolates harboured more than one ESBL; one *E. coli* isolate carried the $bla_{\text{TEM-3}}$, $bla_{\text{SHV-18}}$, and $bla_{\text{CTX-M-14}}$ genes, and two isolates (*E. coli* and *E. cowanii*) carried the $bla_{\text{TEM-3}}$ gene in association with $bla_{\text{CTX-M-14}}$ and $bla_{\text{SHV-18}}$ genes, respectively. In 12 isolates [*E. coli* (n=3); *Enterobacter* spp. (n=3); *Serratia* spp. (n=3); *R. aquatilis* (n=2); and *P. mirabilis* (n=1)] ESBL genes in association with broad-spectrum β-lactamases were detected (Figure 4.1).

AmpC resistance genes were detected in 18/58 (31 %) isolates harbouring β-lactamase genetic determinants (Figure 4.1). In 17 isolates, only one pAmpC genetic determinant was detected;

bla$_{MIR-20}$ (n=4), *bla*$_{MIR-16}$ (n=3), *bla*$_{ACT-58}$ (n=2), and one isolate each carried *bla*$_{CMY-2}$, *bla*$_{MIR-14}$, *bla*$_{ACT-29}$, *bla*$_{ACT-10}$, *bla*$_{ACT-2}$, *bla*$_{EC}$, *bla*$_{CMY-161}$, or *bla*$_{CMY-87}$ respectively. Among these 17 isolates, five isolates [*Enterobacter* spp. (n=2), *E. coli* (n=1), *R. aquatilis* (n=1), and *S. fonticola* (n=1)] also harboured ESBL genetic determinants. One *Proteus penneri* isolate carried three AmpC genes (*bla*$_{ACT10}$, *bla*$_{DHA-18}$, and *bla*$_{CMY-49}$). The EBC family of the AmpC genetic determinants was the most dominant type.

4.4 Discussion

Multidrug resistant ESBL/AmpC-producing Enterobacteriaceae were detected, for the first time, in raw vegetables retailed at selected sites in Gauteng Province. Antibiotic resistant opportunistic pathogens on fresh produce are a serious health concern that contributes towards the burden of AMR in different environments leading to increased risk of infection if colonization in humans occurs (Al-Kharousi et al., 2016). Enterobacteriaceae regarded as emerging bacterial threats include *E. coli*, *K. pneumoniae* and *Enterobacter* spp. showing resistance to β-lactams and aminoglycosides (Fair and Tor, 2014). Presumptive ESBL-producers, predominantly *E. coli*, *K. pneumoniae, E. cloacae and E. asburiae*, were detected in 17.4 % of our vegetable samples analysed. This is lower than the 25.4 % reported by Zurfluh et al. (2015) for imported vegetables into Switzerland from the Dominican Republic, India, Thailand, and Vietnam, but higher than the 6 % reported by Reuland et al. (2014) on retail vegetables in the Netherlands. Similar to Blaak et al. (2014), environmental ESBL-producing Enterobacteriaceae isolated from vegetables included *S. fonticola* and *R. aquatilis*.

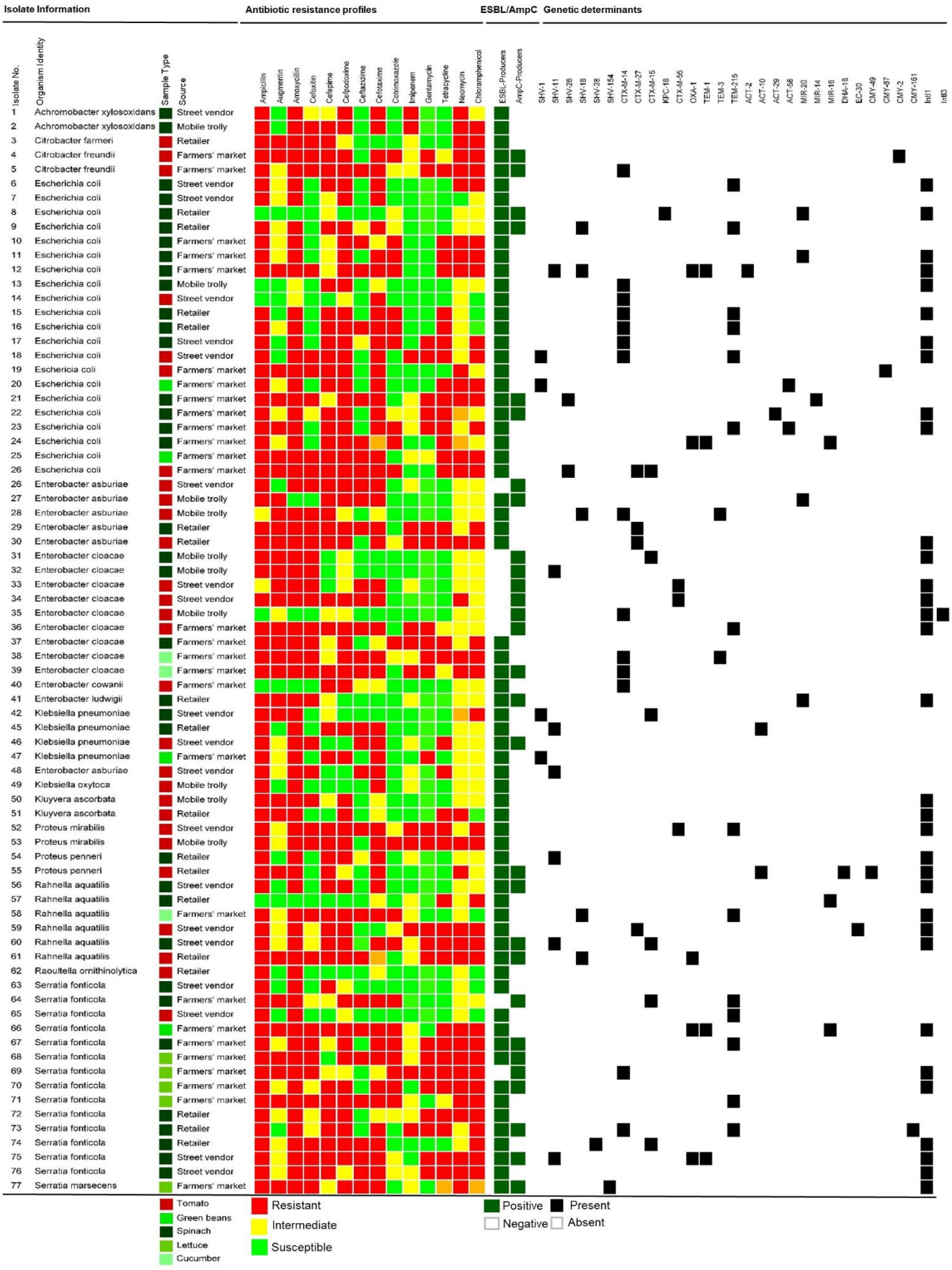

Figure 4.1: A summary of the species isolated from different fresh vegetables purchased from formal and informal markets, indicating the phenotypic resistance profiles and the ESBL/AmpC genetic determinants detected.

Phenotypic confirmation of ESBL/AmpC production showed that 61 (79.9 %) of the 77 analysed Enterobacteriaceae isolates displayed an ESBL-producing phenotype and 41.6 % an AmpC-producing phenotype, which is higher than results reported by van Hoek et al. (2015). Isolates with a combined ESBL- and AmpC-producing phenotype were also observed in 35 % of the isolates. MDR phenotypes (resistance to ≥ 3 antimicrobial classes) were observed in 96.1 % of our analysed isolates. The most prevalent non-β-lactam resistance profiles showed resistance against aminoglycoside (94.8 %), chloramphenicol (85.7 %) and tetracycline (53.2 %). This is higher than reports from similar studies which showed resistance to aminoglycosides (46.7 % - 66.7 %), chloramphenicol (33.3 %) (Zurfluh et al., 2015; Ben Said et al., 2016), and tetracycline (46.7 %) (Ben Said et al., 2016) in ESBL-producing Enterobacteriaceae.

Genes expressing broad-spectrum β-lactamases, ESBLs and/or AmpC β-lactamases were detected in 69.9 % of our MDR isolates. Co-expression of ESBL and AmpC genes in environmental (van Hoek et al., 2015; Ye et al., 2017a) and clinical (Tau et al., 2012; Kharat et al., 2017) Enterobacteriaceae isolates have also been reported. Globally the *bla*$_{\text{CTX-M-type}}$ ESBL genes are predominant in Enterobacteriaceae, which was similar in our study, the majority detected in *E. coli* isolates. *bla*$_{\text{CTX-M-14}}$ was the main genetic determinant detected from mostly *E. coli* and *C. freundii* isolates, which corresponds to results obtained from vegetable samples in Tunisia (Ben Said et al., 2016). Isolates harboring *bla*$_{\text{CTX-M-15}}$ included *E. coli*, *E. cloacae*, *K. pneumoniae*, *R. aqualtilis*, and *S. fonticola* and were second most prevalent in our study.

The *bla*$_{\text{CTX-M-15}}$ genetic determinant was the most prevalent gene detected in *E. coli* and *K. pneumoniae* isolates from fresh vegetables imported into Switzerland from India and the Dominican Republic (Zurfluh et al., 2015). This is in agreement with reports that *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-15}}$ are predominant and have been associated with clinically relevant

Enterobacteriaceae infections (Ehlers et al., 2009; Zurfluh et al., 2015). In contrast to Njage and Buys (2014), who predominantly detected *bla*$_{\text{CTX-M Group 8/25}}$ positive *E. coli* isolates from lettuce in the North West Province (SA), no *bla*$_{\text{CTX-M Group 8/25}}$ genes were detected in any of our *E. coli* isolates from the vegetable samples analysed. The *bla*$_{\text{CTX-M-15}}$ (CTX-M Group 1) and *bla*$_{\text{CTX-M-14}}$ (CTX-M Group 9) genes detected in our environmental isolates, reported to be closelely related to chromosomally encoded *bla*$_{\text{FONA}}$ and *bla*$_{\text{RAHN}}$ genes of *S. fonticola* and *R. aquatilis*, had no significant similarity in the GenBank database using NCBI BLAST based on total BLAST alignment scores. This contrasts results reported by Raphael et al. (2011) where sequences similar to *bla*$_{\text{RAHN-2}}$ and *bla*$_{\text{FONA-5}}$ was detected using *bla*$_{\text{CTX-M}}$ primers.

In our study, five isolates including *E. coli*, *Enterobacter* spp., *R. aquatilis*, and *S. fonticola* simultaneously harboured ESBL and AmpC genes. Environmental isolates are known to carry chromosomally encoded AmpC β-lactamases. However, Enterobacteriaceae harbouring both chromosomal and pAmpC β-lactamases are increasingly reported to hydrolyze broad-spectrum cephalosporins more efficiently, resulting in adverse treatment options in clinical settings (Jacoby, 2009; Reuland et al., 2014). The 18 isolates in which pAmpC resistance genes were detected, predominantly included the EBC type pAmpC β-lactamases (identified as *bla*$_{\text{ACT}}$/*bla*$_{\text{MIR}}$). This contrasts with two previous studies where *bla*$_{\text{CIT}}$, *bla*$_{\text{DHA}}$, or *bla*$_{\text{ACC}}$ pAmpC β-lactamases were mostly detected in Enterobacteriaceae isolated from fresh produce and water samples (Njage and Buys, 2014; Ye et al., 2017a). The *bla*$_{\text{ACT/MIR}}$ genes have been reported to be the dominant AmpC genetic determinants in *Enterobacter* spp. causing intra-abdominal infections (Khari et al., 2016) and were detected in seven of the *Enterobacter* spp. isolates in our study. The fact that fresh produce can serve as a reservoir of MDR ESBL/AmpC-producing Enterobacteriaceae, including their genetic determinants, constitute a potential health risk to the consumer as resistance to antimicrobials frequently used to treat human infections were shown.

4.5 Conclusion

For the first time, the presence of multidrug resistant ESBL/AmpC producing Enterobacteriaceae isolated from raw vegetables sold at selected formal and informal retailers in Gauteng Province, South Africa were shown. The results obtained from screening at these selected sites indicate that further investigation of different fresh produce types in Gauteng and other provinces in SA is also necessary. Future studies should focus on surveillance of fresh produce production systems from farm to retail to identify potential sources of contamination which contribute to the presence and dissemination of antimicrobial resistant microorganisms and their genetic determinants and will be addressed in Chapter 5 and Chapter 6. Since AR is a worldwide problem, a global solution is required that integrates the contributions from governmental departments as well as from the scientific community.

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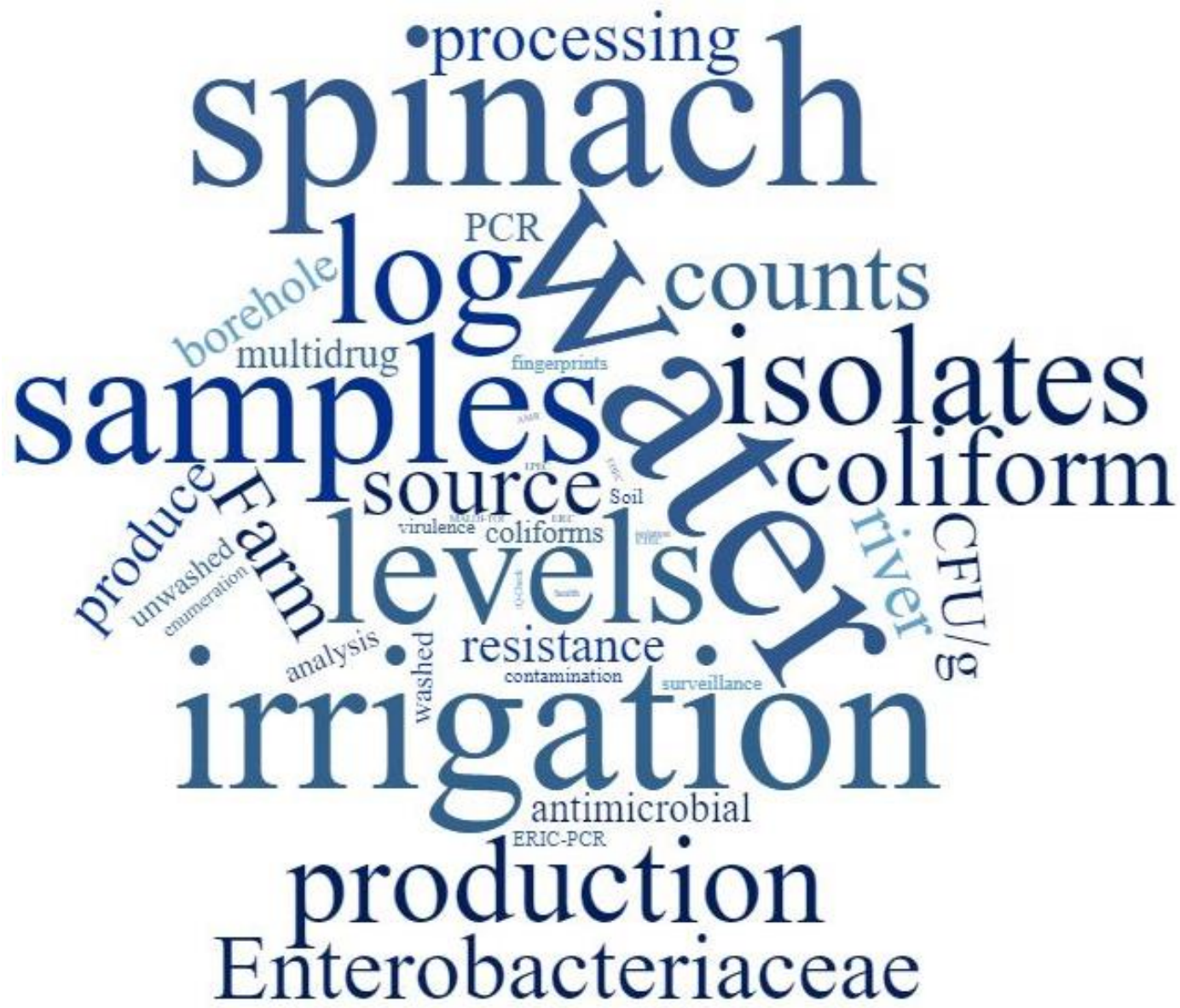
Chapter 5

"Water is our most precious and interconnected natural resource. It sustains all ecosystems, communities, and economies from local watersheds to the seas. It's vital to sustaining our health, safety and the environments in which we live and work. Simply put, water is life." -*Alexandra Cousteau*



Commercial Spinach Supply Chains

Farm Processing Retail

288 Samples

Microbiological safety, antimicrobial resistance and source-tracking of isolated *Escherichia coli*

***Escherichia coli* was isolated from 22.57% (n=65/288) of all samples, *Salmonella* spp. from four water samples, and no *Listeria monocytogenes* was detected.**

- *E. coli* enumerated throughout the chain where river water was directly used for overhead irrigation at levels between 0.00-3.22 log CFU/g.
- The wash water during processing in both production scenarios had acceptable *E. coli* levels according to the international guidelines.
- *E. coli* enumerated from 8.33% of the spinach samples only.

- Generic *E. coli* isolated from 40.30% water and 14.60% spinach samples.
- 80 characterised *E. coli* isolates, 43.75% (n=35) were multidrug resistant.
- More antibiotic resistant *E. coli* isolates detected from irrigation water (52.5%) than from spinach (37.5%).

ERIC-PCR profiles: high similarity values (>90.0 %) for irrigation water and spinach *E. coli* isolates at different points of production, processing or retail in each of the respective supply chains.

The necessity of using clean and safe irrigation water was highlighted with the need for standardised risk-based microbiological safety parameters for irrigation water of ready-to-eat fresh vegetables.

Richter, L., du Plessis, E.M., Duvenage, S. & Korsten, L. (2021) Microbiological safety of spinach throughout commercial supply chains in Gauteng Province, South Africa and characterization of isolated multidrug-resistant Escherichia coli. Journal of Applied Microbiology, 00, 1–21. https://doi. org/10.1111/jam.15357

Plant to Food

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Microbiological safety, phenotypic and genotypic characterisation of multidrug resistant *Escherichia coli* isolated throughout commercial spinach supply chains in Gauteng Province, South Africa[5]

Abstract

The microbiological quality and human foodborne pathogen presence from spinach production systems from farm-to-sale, as well as phenotypic and genotypic characteristics of isolated *Escherichia coli* were investigated. Samples (n=288) were collected from two commercial supply chains using either river or borehole water for irrigation. *Escherichia coli* was enumerated throughout the chain where river water was directly used for overhead irrigation at levels between 0.00-3.22 log CFU/g. Mean Enterobacteriaceae and coliform counts of spinach ranged between 3.33-6.57 log CFU/g and 3.33-6.64 log CFU/g, respectively. Following enrichment, isolation and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) identification, *E. coli* was isolated from 22.57% (n=65/288) of all samples, *Salmonella* spp. from four water samples, and no *Listeria monocytogenes* was detected. Of the 80 characterised *E. coli* isolates, one harboured the *stx2* virulence gene, whilst 43.75% (n=35) were multidrug resistant. Source tracking showed a connection between *E. coli* in source water and on the irrigated crop using enterobacterial repetitive intergenic consensus (ERIC)-PCR analysis. The importance of compliance of irrigation water microbiological guidelines for vegetables consumed raw was highlighted, since the similarity of *E. coli* isolates demonstrated transfer from irrigation water to spinach in both scenarios. Multidrug resistant *E. coli* presence throughout spinach production emphasises the necessity of environmental surveillance

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programs as part of a one health approach to develop antimicrobial resistance mitigation strategies.

5.1 Introduction

Enterobacteriaceae colonize the gastrointestinal tracts of humans and animals. Moreover, members of this family form part of the concept of microbiological criteria commonly used to assess hygiene standards and is often linked to safety of food products, including fresh produce (Rajwar et al., 2015). Although most fresh vegetables carry epiphytic microorganisms, contamination with potential human pathogenic bacteria (including pathogenic *Escherichia coli* and *Salmonella* spp.) may arise throughout production and processing of fruit and vegetables. This follows as manure-amended soil, contaminated irrigation water, and different handling practices are often used in fresh produce production, and the ability of pathogens to persist and proliferate in vegetables (Tope et al., 2016).

Surveillance of foodborne pathogens form an important part of disease outbreak assessment and is a critical component of food safety. However, foodborne diseases in South Africa (SA) are often not reported in an epidemiological surveillance system- or are under-reported and poorly investigated (Frean, 2010; Bisholo et al., 2018). Globally, an increase in foodborne outbreaks linked to fresh produce have been reported, with leafy green vegetables in particular posing a higher risk for the consumer [World Health Organisation (WHO), 2008]. Leafy green vegetables often associated with foodborne illness include spinach, lettuce and kale [Centre for Disease Control and Prevention (CDC), 2017; European Food Safety Authority (EFSA), 2018]. Sources of contamination with pathogens such as *E. coli* O157:H7 or *Listeria monocytogenes* in leafy green vegetables include contaminated irrigation water, soil or processing facilities (Self et al., 2019; CDC, 2020). Specific examples in the United States of America (USA) include the 2006 multistate packaged spinach outbreak and the 2019 multistate romaine lettuce

outbreak, both associated with *E. coli* O157:H7, whilst in 2016 a multistate outbreak in packaged leafy green salads associated with *L. monocytogenes* were reported (Jay et al., 2007; Self et al., 2019; CDC, 2020).

Irrigation water is regarded as one of the primary reservoirs, and routes of transmission, of human pathogenic bacteria onto fresh produce during primary production (Allende and Monaghan, 2015). In SA, 25 – 30% of the agricultural industry relies on irrigation, with the total volume of water utilised for irrigated agriculture estimated to be between 51% and 63% of total water available in the country (Bonthuys, 2018). Sources of irrigation water include untreated or treated wastewater, surface water, borehole water from shallow- or deep groundwater and potable or rainwater (Iwu and Okoh, 2019). The water scarcity in SA has led to the use of mainly surface water for irrigation purposes in vegetable production (Du Plessis et al., 2015). The microbiological quality of surface water are severely compromised due to mainly densely populated human settlements close to the surface water sources as well as mining and industry activities (Oberholster and Botha, 2014; Du Plessis et al., 2015; Duvenage and Korsten, 2017; Iwu and Okoh, 2019). As fresh produce production and processing rely on potable water, increased food safety risks arise when irrigation water are increasingly being polluted (Uyttendaele et al., 2015). The frequency of fresh produce contamination, prevalence of generic *E. coli* levels, and the presence of pathogenic foodborne bacteria in irrigation water may vary (Allende and Monaghan, 2015; Alegbeleye et al., 2018). This follows as seasonality, land use interactions (e.g. waste water treatment plants upstream of irrigation source water) and farming production practices differ (Allende and Monaghan, 2015; Alegbeleye et al., 2018). Wash water is another potential contamination source within fresh produce production. During processing, wash water is often reutilized, resulting in continuous contact of large volumes of produce and potential cross-contamination (Machado-Moreira et al., 2019).

In addition to the prevalence of foodborne pathogens, the need for surveillance of antimicrobial resistance (AMR) in crop production exists. Prevalence of antimicrobial multidrug resistant bacteria isolated from agricultural environments poses an additional potential health threat to consumers (Blaak et al., 2014c; Ben Said et al., 2016; Tope et al., 2016; Ye et al., 2017a). Previous South African studies reported close AMR phenotypic relatedness at a 69% similarity level in *E. coli* isolated from irrigation water and onion samples (Du Plessis et al., 2015), whilst *E. coli* isolates from river water and field cabbage were phenotypically related at a 80% similarity level (Jongman and Korsten, 2016). Njage and Buys (2014), further reported a high degree of genetic relatedness in *E. coli* with similar β-lactamase resistance profiles in isolates from irrigation water and lettuce.

However, no studies have investigated the microbiological quality and presence of antimicrobial resistance in foodborne pathogens throughout fresh produce supply chains including the on-farm environment, harvesting, processing and packaging, up to the point of sale. The aim of this study was to determine the microbiological quality and presence of foodborne pathogens (*E. coli*, *Salmonella* spp. and *L. monocytogenes*) in irrigation water and spinach from farm, through processing up to retail. Furthermore, to characterise the *E. coli* isolated from the respective spinach supply chains phenotypically using antibiotic resistance profiles and genotypically through diarrheagenic gene screening and Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR analysis.

5.2. Materials and Methods

5.2.1 Sampling study areas

Samples were collected from two different commercial spinach production scenarios typically seen in vegetables supply chains in Gauteng Province (Figure 5.1) (Richter et al., 2020). River water was used with overhead irrigation and open field cultivation in the first scenario (Farm

A). Depending on the field layout, river water was either used directly or used after storing in a holding dam. For the second spinach production scenario, two farms were selected from various farms supplying a central processing facility for sampling of baby spinach grown in tunnels using borehole water for irrigation. A comparison of the farms and their practices is given in Table 5.1.

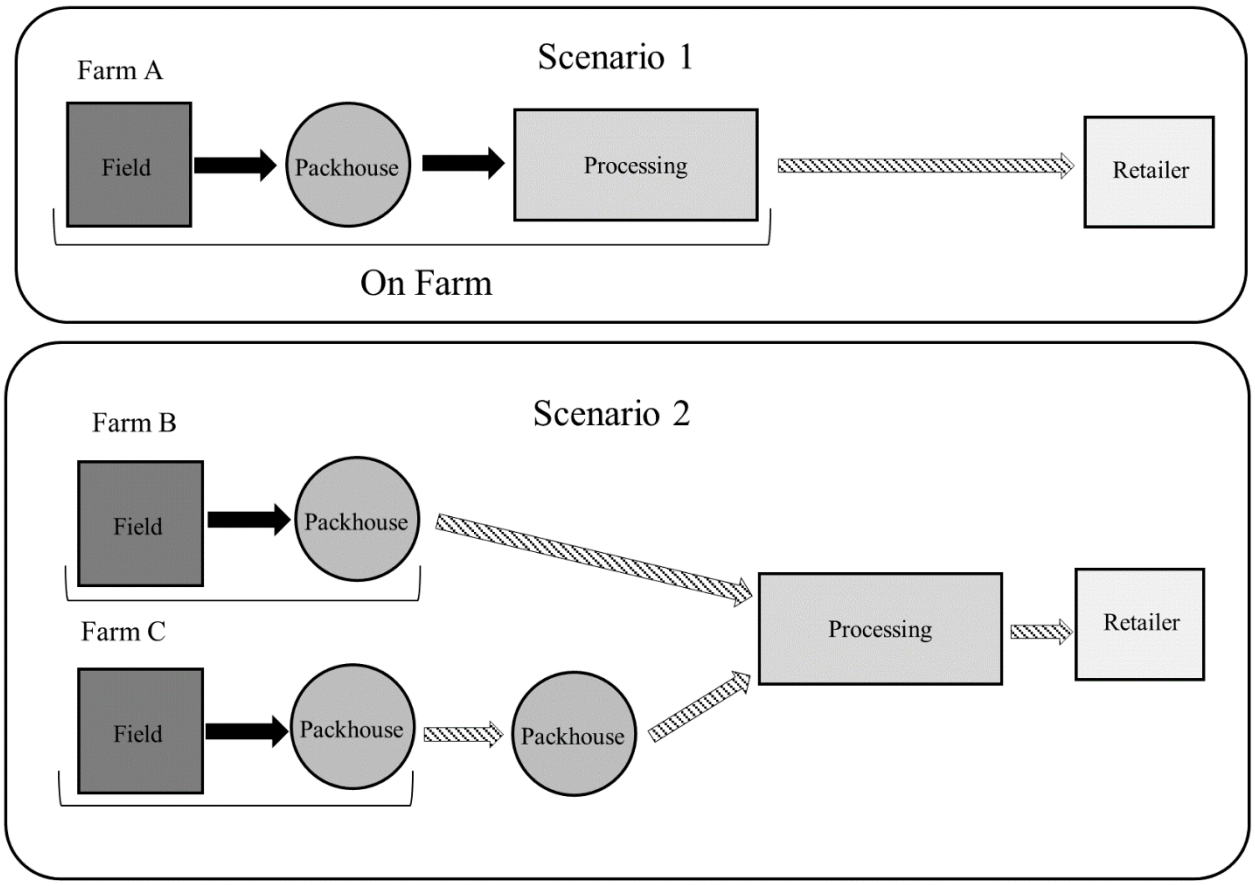

Figure 5.1: Typical spinach production scenarios in Gauteng Province, South Africa. Square brackets show all production practices that occurred on the same farm/premises of each respective scenario. Dashed arrows indicate transportation for processing at a different location and retail of the spinach. In the first scenario, all processing occurred on farm before spinach was transported to commercial retailers or retail distribution centres, whilst a central processing facility was used in the second scenario where supplier farms with different production practices provided the fresh produce.

Table 5.1: Comparison of the processing practices and cultivation of the three spinach farms assessed for this study in 2017

Practice	Farm A (July and November)	Farm B (June and October)	Farm C (July and October)
Certification status	GLOBAL G.A.P., Intertek food management system based on SANS 10049, 150/75 22002, Codex HACCP principles and GFS1	GLOBAL G.A.P., Packing facility: SANS 10330, SANS 10049, R918, The Global Food Safety Initiative, Act 54 of 1972 Act 85, Codex Alimentarius, R692	GLOBAL G.A.P.
Production system	Open field cultivation	Tunnels	Tunnels
Irrigation water source	River, water pumped directly from river or to a storage dam	Borehole water, pumped into a storage dam	Borehole water, pumped into a storage dam
Irrigation water	Uncovered storage dam	Two additional water storage dams (covered with a net) over which the source water is pumped in and circulated	Source water is pumped into another water storage dam
Irrigation method	Overhead irrigation	Overhead irrigation	Overhead irrigation

Postharvest processing of spinach on Farm A included hand picking and making up of spinach bunches in the field. At the packhouse, spinach bunches were then soaked in a wash bath (containing borehole water) to remove excess soil, labelled and stored in a cold room (4°C, ≤ 24h), before transportation to the specific retailers or retailer-distribution centres usually within two days (48h). Additionally, hand harvested spinach leaves in crates were also sorted in the packhouse, where the stalks were cut (by hand) and the leaves were put through a cutting machine, chlorine washed, dried, hand-packed and sealed prior to cold-room storage (4°C, ≤ 24h), before transportation to the specific retailers or retailer-distribution centres within a day (24h).

The baby spinach harvested on Farms B and C were hand sorted along a conveyer belt and packed and weighed in plastic containers in the pack houses on the farm for the unwashed product line, prior to cold-storage and transportation (4°C, ≤ 24h) to the processing facility where it was labelled and distributed to the specific retailers. Additionally, baby spinach leaves harvested in crates were cold-stored (4°C, ≤ 24h) and transported to the processing facility. At the processing facility, the baby spinach leaves from Farms B and C were cold stored no longer than three days (72h), chlorine washed (75 – 80ppm active chlorine), packed, and sealed before transportation to the specific retailers.

5.2.3 Sample collection

A total number of 288 samples were collected at selected sampling points throughout the supply chains from the two spinach production scenarios as previously described (Richter et al., 2020). Soil samples were collected at harvest (n=6 composite samples). Water samples (n=42) were analysed from the source (borehole or river) and irrigation point, as well as treated wash water during processing (n=30). Spinach samples (n=192) included samples taken at harvest, during processing and at retail for each respective farm. Additionally, contact surface swab samples throughout production and processing of the fresh produce (n=18) were also included.

5.2.4. Microbiological analysis

Soil. Soil samples were collected from five replicate points during harvest from the spinach production fields. A composite sample of 25g (5g from each replicate) were added to 225ml 3M buffered peptone water (BPW) (3M Food Safety, Minnesota, USA), from which a tenfold dilution series of each soil sample was prepared and plated in duplicate onto *E. coli*/ coliform count plates (3M Petrifilm, 3M, St. Paul, Minnesota, USA) for hygiene indicator bacteria enumeration, (coliforms, *E. coli*) and on Violet Red Bile Glucose (VRBG) (Oxoid,

Basingstoke, UK) agar plates for Enterobacteriaceae enumeration following incubation for 24h at 37 °C (Du Plessis et al., 2015; van Dyk et al., 2016).

The remaining BPW-sample mixture was incubated for 24h at 37°C for detection of *E. coli* and *Salmonella* spp. After incubation, the BPW-sample mixtures were subsequently streaked (10µl) onto Eosin methylene blue (EMB) media (Oxoid) for the detection of *E. coli*. The presence of *Salmonella* spp. was assessed using the iQ-Check *Salmonella* II Kit AOAC 010803 (BioRad, Johannesburg, SA) according to the manufacturer's instructions. Once positive results were obtained, the sample was streaked onto Xylose lysine deoxycholate (XLD) agar (Biolabs, Johannesburg) and *Salmonella* Brilliance agar (Oxoid) and incubated for 24h at 37°C. The presence of *Listeria* spp. was assessed by incubating an additional 25g of each sample in 225ml Buffered *Listeria* Enrichment Broth (BLEB) (Oxoid) at 30°C and subsequently using the iQ-Check *Listeria monocytogenes* II Kit AOAC 010802 (BioRad) according to the manufacturer's instructions. Once positive results were obtained, the sample was streaked onto Agar Listeria Ottavani and Agosti (ALOA) (Biomèrieux, Johannesburg) and Rapid'L.mono agar (BioRad) and incubated for 48h at 37°C.

Water. Water (100ml and 1L) samples were collected in triplicate from each sampling point (source, irrigation pivot point and wash water). According to the manufacturer's instructions, the 100ml water samples were used for enumeration of coliforms and *E. coli* using the most probable number (MPN) with Colilert-18 (IDEXX Laboratories Incorporated, Westbrook, ME, USA) reagents heat sealed in a Quanti-Tray/2000 (IDEXX). The trays were incubated at 37°C for 24h and inspected for chromogenic reactions and fluorescence indicating the presence of coliforms and *E. coli,* respectively. The results were recorded as log MPN *E. coli*/100 ml and log MPN coliforms/100ml. From the 1L water samples, 1ml was used to conduct a serial dilution in 9ml 0.1 % BPW, with a 100µl aliquot from each serial dilution (ranging from 10^{-1}

– 10^{-4}) plated in duplicate onto VRBG (Oxoid) agar plates for enumeration of Enterobacteriaceae.

The remaining 1L water samples were filtered through a 0.45µm nitrocellulose membrane (Sartorius, Johannesburg). The membrane was subsequently placed into 50 ml BPW and incubated for 24h at 37°C for detection of foodborne pathogens (*E. coli*, *Salmonella* spp. and *Listeria* spp.). Following enrichment, the same detection methods as described for the soil samples were conducted for the water samples.

Fresh produce. After removal of the spinach stalks, at least three leaves were used to prepare 50g composite samples. For the baby spinach, 50g composite samples were obtained. Each sample was aseptically cut and placed into a sterile polyethylene strainer stomacher bag (Seward Ltd., London, UK) containing 200ml (3M, Johannesburg) BPW in a 1:4 weight to volume ratio. Individual vegetable samples were blended for 5min at 230rpm in a Stomacher® 400 Circulator paddle blender (Seward Ltd., London, UK). To enumerate hygiene indicator bacteria (coliforms and *E. coli*), a tenfold dilution series of each BPW sample was made in duplicate, plated onto *E. coli*/coliform count plates and incubated for 24h at 37 °C according to the manufacturer's instructions (3M Petrifilm, 3M, St. Paul, Minnesota, U.S., ISO method 4832). Enterobacteriaceae were enumerated by plating 100 µl of the dilution series in duplicate onto VRBG agar plates and incubated for 24 h at 37°C (Oxoid). The remaining BPW samples were incubated for 24h at 37°C and after enrichment, detection of foodborne pathogens was conducted as described for the soil samples.

Contact surfaces. Transystem™ swabs with Amies medium (Lasec, Johannesburg) were used to sample a 25cm^2 area from crates, tables and conveyer belt surfaces respectively, in triplicate, according to the standard procedures for environmental swab sampling (Public Health England, 2014). The swab samples were added to 9ml 3M BPW for enumeration of coliforms/*E. coli*

and Enterobacteriaceae as described for the soil samples. The swab samples were subsequently enriched for 24h at 37°C in BPW. Detection and isolation of *E. coli*, *Salmonella* spp. and *Listeria* spp. were done as described for the soil samples.

All presumptive positive *E. coli*, *Salmonella* spp. and *Listeria monocytogenes* colonies from the soil, water, spinach, and contact surface samples were isolated and purified. Isolates were identified using matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker, Bremen, Germany) to species level as described by Standing et al. (2013) and AOAC-OMA#2017.09. Briefly, the purified presumptive positive colonies were regrown in 9 ml tryptone soy broth (TSB) (MERCK, Johannesburg) and incubated overnight at 37°C. Subsequently, isolates (10µl) were streaked out on Nutrient Agar (MERCK) and the plates were incubated overnight at 37°C and subjected to the MALDI Biotyper protocol (Bruker) (Standing et al., 2013) (Appendix D Table D10). All strains were tested in duplicate.

5.2.5 Antimicrobial susceptibility testing

The *E. coli* isolates (n=80) from the different spinach production scenarios were further tested for antimicrobial resistance against seven antibiotic classes. The Kirby-Bauer disk diffusion technique was used to determine the resistance patterns of the isolates [Clinical Laboratory Standard Institute (CLSI), 2018]. Briefly, each isolate was cultured in 9ml TSB and incubated for 24h at 37 °C. Of each TSB sample, 100µl was subsequently inoculated into 9ml brain heart infusion (BHI) broth (MERCK) and incubated for 24h at 37°C. A 120 µl bacterial suspension was then plated onto Mueller-Hinton agar plates (MERCK) and screened for resistance against 11 antibiotics belonging to seven classes. (Mast Diagnostics, Bootle, UK, supplied by Davies Diagnostics, Midrand, SA) using the Disk Master Disc dispenser (Mast Diagnostics, Bootle, UK), and incubated for 16-18hr at 37°C. Antibiotics screened for included ampicillin-10µg, amoxicillin-clavulanic acid-20µg/10µg, amoxicillin-10µg, trimethoprim-

sulfamethoxazole/cotrimoxazole-1.25µg/23.75µg, cefoxitin-30µg, cefepime-30µg, imipenem-10µg, neomycin-10µg, tetracycline-30µg, gentamycin-10µg, and chloramphenicol-30µg (Mast Diagnostics, Randburg, SA) (CLSI, 2018). Breakpoints were then compared to (CLSI, 2018) and isolates resistant to three or more antimicrobial classes were regarded as multidrug resistant. *Escherichia coli* ATCC 25922 was included as a control (CLSI, 2018).

5.2.6 Molecular characterisation of diarrheagenic *Escherichia coli*

The presence of different diarrheagenic *E. coli* virulence genes for enterotoxigenic *E. coli* (ETEC) (*lt* and *st* genes), enteropathogenic *E. coli* (EPEC) (*bfpA* and *eaeA* genes), enteroaggregative *E. coli* (Eagg) (*eagg* gene), enterohaemorrhagic *E. coli* (EHEC) (*eaeA*, *stx1* and *stx2* genes), and enteroinvasive *E. coli* (EIEC) (*ipaH* gene) were analysed by PCR and sequencing, with the *mdh* gene used as internal control in all reactions (Table 5.2) (Omar and Barnard, 2010a). Control strains for the PCR reactions included DSM 10973 and DSM 27503 (ETEC); DSM 8703 and DSM 8710 (EPEC); DSM 27502 (Eagg); *E. coli* O157:H7 (EHEC); and DSM 9028 and DSM 9034 (EIEC) and ATCC 25922.

Single colonies of each *E. coli* isolate were cultured aerobically under shaking conditions at 200rpm in tryptone soy broth (TSB) (MERCK) for 24h at 30°C. The cells were pelleted by centrifugation (12,500*g* for 10min), DNA was extracted using the Quick-gDNA Mini-Prep kit (Zymo Research, Irvine, USA) and the DNA concentration was determined using the Qubit dsDNA Broad Range Assay and a Qubit 2.0 fluorometer (Life Technologies, Johannesburg). PCR was performed using 1x DreamTaq Green PCR Master Mix (ThermoFisher Scientific, Johannesburg), with specific primers, and thermocycling conditions for each of the genes as described in Table 5.2.

Table 5.2: Primers used for screening of diarrheagenic *Escherichia coli* isolated from fresh produce sold formally and informally

Diarrheagenic *Escherichia coli*	**Target genes**	**Primer sequences (5'-3')**	**Thermocycling conditions**	**Expected amplicon size (bp)**	**Reference**
Enterotoxigenic	*Lt*	F: GGC GAC AGA TTA TAC CGT GC R: CGG TCT CTA TAT TCC CTG TT	95°C, 15min; 35 cycles of 94°C, 45s; 55°C, 45s; 68°C, 2.5 min; 72°C 5 min	410	Omar and Barnard, 2010
	St	F: TTT CCC CTC TTT TAG TCA GTC AAC TG R: GGC AGG ATT ACA ACA AAG TTC ACA		160	Omar and Barnard, 2010
Enteropathogenic	*bfpA*	F: AAT GGT GCT TGC GCT TGC TGC R: GCC GCT TTA TCC AAC CTG GTA	94°C, 5min; 35 cycles of 94°C, 40s; 68°C, 60s; 72°C, 2min; 72°C 5 min	324	López-Saucedo et al., 2003
	eaeA	F: CTG AAC GGC GAT TAC GCG AA R: GAC GAT ACG ATC CAG	95°C, 15min; 35 cycles of (94°C, 45s; 55°C, 45s; 68°C; 2min	917	Omar and Barnard, 2010
Enteroaggregative	*Eagg*	F: CTG GCG AAA GAC TGT ATC AT R: AAT GTA TAG AAA TCC GCT GTT	94°C, 5min; 35 cycles of 94°C, 40s; 57°C, 60s; 72°C, 2min; 72°C, 5 min	630	Aslani et al., 2011
	Eagg	F: CTG GCG AAA GAC TGA ATC AT R: CAA TGT ATA GAA ATC CGC TGT T	94°C, 5min; 35 cycles of 94°C, 40s; 53°C, 60s; 72°C, 1min; 72°C, 5min	630	Aslani et al., 2011
Enterohemorrhagic	*eaeA*	F: CTG AAC GGC GAT TAC GCG AA R: GAC GAT ACG ATC CAG	95°C, 15min; 35 cycles of 94°C, 45s; 55°C, 45s; 68°C; 2min	917	Omar and Barnard, 2010
	stx1	F: ACA CTG GAT GAT CTC AGT GG R: CTG AAT CCC CCT CCA TTA TG	95°C, 15min; 35 cycles of 94°C, 45s; 55°C, 45s; 68°C; 2min	614	Omar and Barnard, 2010
	stx2	F: CCA TGA CAA CGG ACA GCA GTT R: CCT GTC AAC TGA GCA CTT TG		779	Omar and Barnard, 2010
Enteroinvasive	*ipaH*	F: GTT CCT TGA CCG CCT TTC CGA TAC CGT C R: GCC GGT CAG CCA CCC TCT GAG AGT AC	95°C 5min 35cycles of 95°C 60s; 60°C 90s; 72°C 2min 72°C 10 min	600	Aranda et al., 2004
E. coli	*Mdh*	F: GGT ATG GAT CGT TCC GAC CT R: GGC AGA ATG GTA ACA CCA GAG T	Used as internal control in all abovementioned reactions	304	Omar and Barnard, 2010

5.2.7 Genomic fingerprinting of *Escherichia coli* by repetitive PCR

The same *E. coli* isolates analysed for antimicrobial susceptibility and virulence genes were used to conduct repetitive PCR through generation of Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR fingerprints from each individual spinach production scenario. PCR was performed using 1x DreamTaq Green PCR Master Mix (ThermoFisher Scientific), 80-100ng template DNA and 4µM of each primer in a total reaction volume of 25µL. The forward and reverse primer sequences used to generate the DNA fingerprints were 5'-ATGTAAGCTCCTGGGGATTCAC-3' and 5'-AAGTAAGTGACTGGGTGAGCG-3', respectively (Soni et al., 2014). The PCR conditions were: 95 °C for 4min, followed by 30 cycles of 94°C for 30s, 40°C for 1min and 72°C for 8min, with a final elongation step at 72°C for 15min. The PCR amplicons were visualised in a 2% agarose gel and band patterns were analysed and compared using Bionumerics 7.6 fingerprint analyst software (Applied Maths, Saint-Marten-Latem, Belgium). The percent similarities of digitized bands were calculated using the Pearson's correlation coefficient and the unweighted pair group method with arithmetic mean, and complete linkage alogrithms were used to derive a dendrogram.

5.2.8 Statistical analysis

Data were analysed using SAS version 9.3 statistical software (SAS/STAT User's Guide 1999). A separate analysis of variance (ANOVA) was done for each sampling type to test for significant differences between sampling points (sources) and trip (a repeated measurement over time) was added as a sub-plot factor in the ANOVA. The Shapiro-Wilk test was performed on the standardised residuals to test for deviations from normality (Shapiro and Wilk, 1965). Student's protected t-LSD (Least significant difference) was calculated at a 5% significance level to compare means of significant source effects (Snedecor and Cochran, 1980).

5.3 Results

5.3.1 Microbiological quality analysis

The Enterobacteriaceae, coliform and *E. coli* counts of the irrigation water, wash water and spinach from the farm, through processing and at the retailer from Farm A, Farm B and Farm C are shown in Figure 5.2, Figure 5.3 and Figure 5.4, respectively. The composite soil samples of the three farms had similar mean Enterobacteriaceae and coliform counts, ranging between 3.29-5.22 log CFU/g and 3.05-5.19 log CFU/g respectively, with no *E. coli* enumerated from soil on any of the farms, shown in Appendix D Table D9.

Enterobacteriaceae counts in river water from Farm A ranged from 2.84-3.20 log CFU/ml, while the holding dam and irrigation pivot point counts ranged from 1.61-3.78 log CFU/ml and 0.00-3.83 log CFU/ml, respectively. The trip by source interaction of Enterobacteriaceae counts from water sources on Farm A were not significantly different (p=0.0936) (Appendix D Table D1). However, the Enterobacteriaceae levels were significantly different based on the source of the water (p=0.0083), with river water significantly higher than the dam reservoir and irrigation water in Trip 1. Enterobacteriaceae counts on spinach samples from Farm A were not significantly different (trip x source – p=0.1627, trip – p=0.3639, source – p=1.1646). The Enterobacteriaceae counts on spinach from Farm A ranged from 0.00-6.52 log CFU/g.

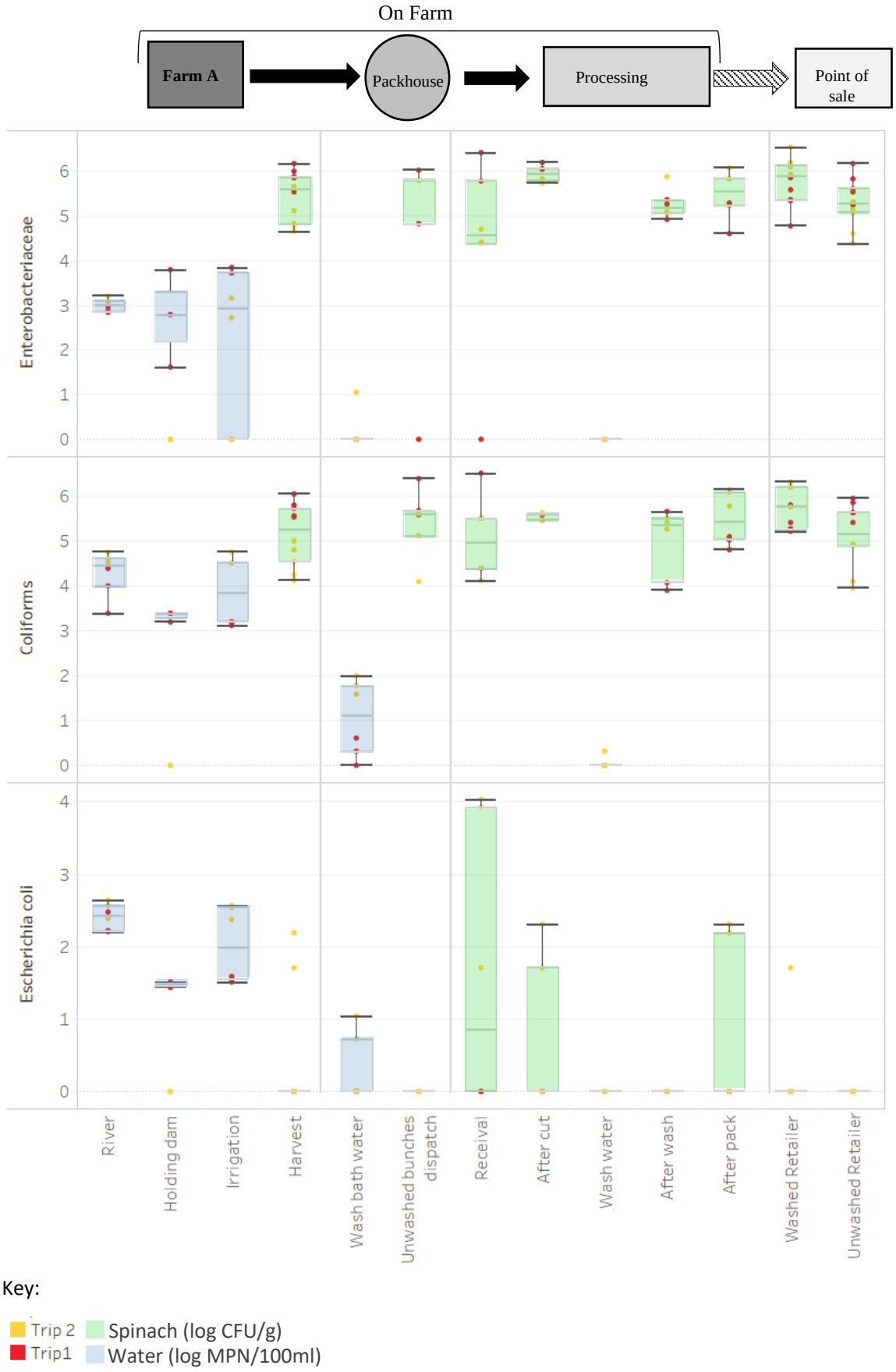

Figure 5.2: Indicator bacteria levels from water (log MPN/100ml) and spinach (log CFU/g) from farm to retail in a spinach production system using river water for irrigation.

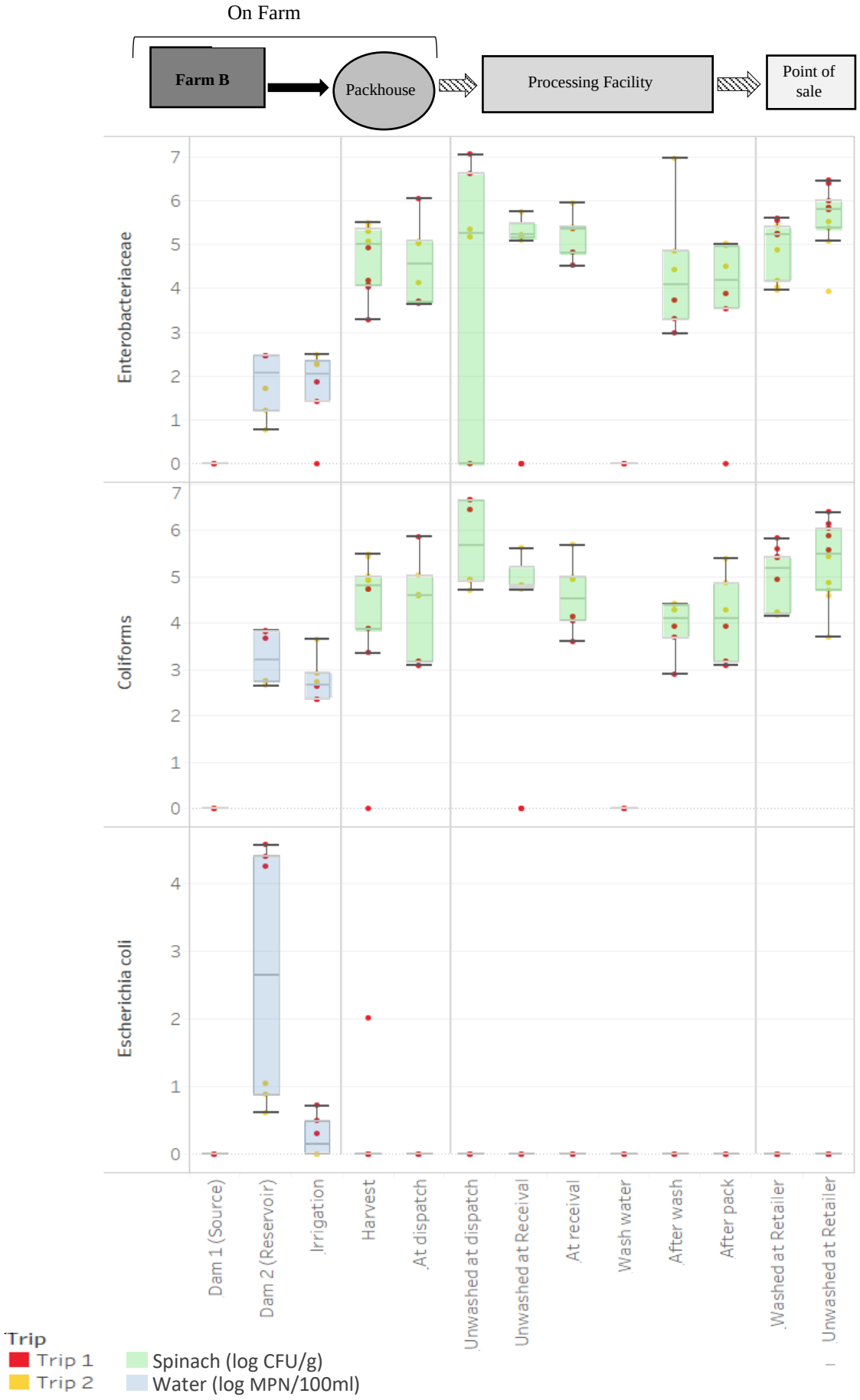

Figure 5.3: Indicator bacteria levels from water (log MPN/100ml) and spinach (log CFU/g) from farm to retail in a spinach production system using borehole water for irrigation and produce were processed at a centralised processing facility.

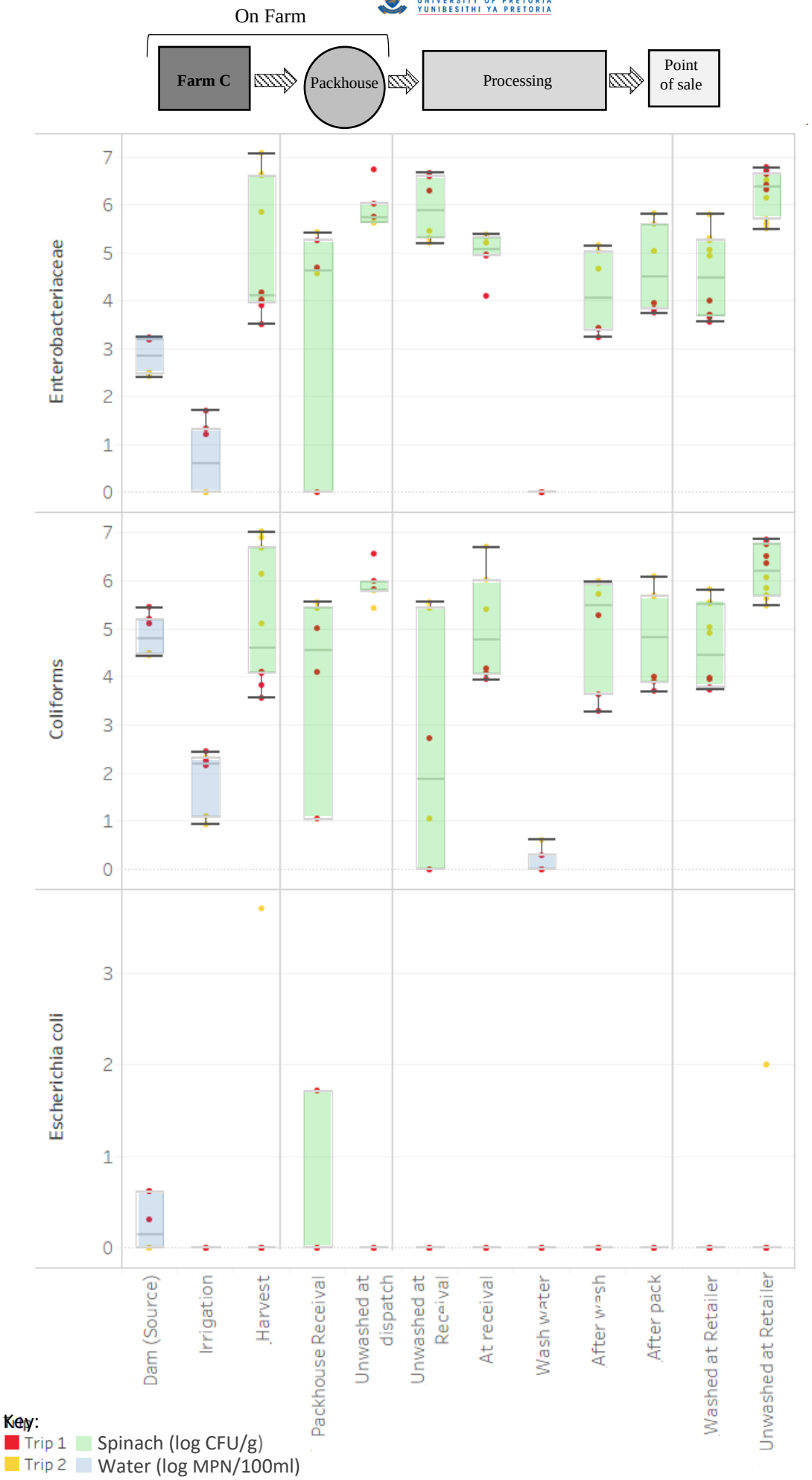

Figure 5.4: Indicator bacteria levels from water (log MPN/100ml) and spinach (log CFU/g) from farm to retail in a spinach production system using borehole water for irrigation and produce were processed at a centralised processing facility.

The coliform levels of river, holding dam and irrigation pivot point water samples from Farm A ranged from 3.38-4.76 log MPN/100ml, 3.19-3.38 log MPN/100ml and 3.11-4.76 log MPN/100ml, respectively. Samples collected from river water during Trip 1 exhibited higher coliform counts than the holding dam and irrigation pivot point water samples during the same trip (p=0.0077) (Appendix D Table D1). River and irrigation pivot point water coliforms levels were not significantly different in Trip 2, and the levels in the borehole water used during processing were significantly lower than the river water levels (p=0.0077). The coliform levels on spinach from Farm A ranged from 3.90-6.50 log CFU/g. Neither trips showed a significant difference (p=0.0003) in coliform levels on unwashed spinach bunches from harvest, in the packhouse, or subsequent retailer samples (Appendix C Table C2). Coliform levels on spinach at harvest, at dispatch, at receival and retailed bunches were all significantly lower during Trip 2 when compared to Trip 1 (p=0.0003) (Appendix C Table C2). The coliform levels on spinach after wash and spinach after pack from Trip 1 was significantly lower than during Trip 2 (p=0.0003). The Trip 1 spinach coliform levels were significantly lower after washing, in comparison to the at harvest, packhouse receival and after cut spinach samples, however the coliform levels of the ready-to-eat (RTE) spinach samples were not significantly different to the harvested spinach (p=0.0003).

Escherichia coli levels in river water ranged from 2.20-2.64 log MPN/100ml, in the holding dam water from 1.43-1.50 log MPN/100ml and in the irrigation pivot point water from 1.50-2.56 log MPN/100ml. These *E. coli* levels were lower than the national regulation limits (<1000 *E. coli*/100ml) for irrigation water [Department of Water Affairs and Forestry (DWAF), 1996]. Similar to the coliform levels, during Trip 1, the river water *E. coli* levels were significantly higher than that of the holding dam and irrigation pivot point water samples (p=0.0257) (Appendix D Table D1). During Trip 2 the *E. coli* levels in the irrigation pivot point water were not significantly different to the river water (p=0.0257), as river water was directly

used for irrigation (Appendix D Table D1). The *E. coli* levels on spinach from Farm A ranged from 0.00-4.03 log CFU/g. The *E. coli* (trip x source) count interactions from spinach were significantly different (p = 0.0012) (Appendix D Table D2). No *E. coli* was enumerated from any of the spinach samples during Trip 1. However, the *E. coli* levels during Trip 2 on spinach at receival were significantly higher (p=0.0012) than spinach after pack, after cut and at harvest, with all other samples having significantly lower *E. coli* levels (p=0.0012) (Appendix D Table D2).

The coliform levels from swab samples throughout processing on Farm A ranged from 2.60-6.32 log CFU/cm^2, with a significant difference between the trip x source interactions (p=0.0021) (Appendix D Table D3). In contrast to the coliform levels from the contact surface swab samples, Enterobacteriaceae levels ranged from 2.70-6.13 log CFU/cm^2, with no significant difference in the trip x source interactions (p=0.1333) (Appendix D Table D3). The *E. coli* levels on the contact surfaces ranged from 0.00-2.74 log CFU/cm^2. Similar to the Enterobacteriaceae counts, the trip x source interactions of *E. coli* from contact surfaces were not significantly different (p=0.3325). The *E. coli* counts on per trip were significantly different (p=0.0034) with Trip 2 having higher levels than Trip 1 (Appendix D Table D3).

The Enterobacteriaceae counts of the borehole water from Farm B were 0.00 log CFU/ml, while the counts of the reservoir dam and irrigation pivot point water samples ranged between 0.78-2.46 log CFU/ml and 0.00-2.49 log CFU/ml, respectively. The Enterobacteriaceae levels of the dam reservoir and irrigation pivot point water increased significantly when compared to the borehole source water (p=0.0365) (Appendix D Table D4). Additionally, the trip independently demonstrated significant differences with Trip 2 having higher Enterobacteriaceae counts than Trip 1 (p=0.0058) (Appendix D Table D4). The Enterobacteriaceae counts on spinach from Farm B ranged between 0.00-7.05 log CFU/g

(Figure 5.3), with a significant difference (p=0.0006) in the trip x source interactions (Appendix D Table D5).

The coliform counts of the borehole water were < limit of detection (LoD) (5 MPN/100ml), while the coliform counts from the reservoir dam and irrigation pivot point water samples ranged between 2.65-3.84 log MPN/100ml, and 2.35-3.64 log MPN/100ml, respectively (Figure 5.3). The coliform counts were significantly different (trip x source interactions p=0.0074) (Appendix D Table D4). Coliform counts on spinach from Farm B ranged between 0.00-6.65 log CFU/g (Figure 5.3), with significant differences observed (trip x source interactions p=0.0002). Additionally, the coliform counts on the spinach samples from the different points throughout processing had significant differences (p=0.0037) with significantly higher coliform counts on spinach at retailer samples than that of the washed spinach samples at the processing facility (Appendix D Table D5).

Escherichia coli counts in irrigation water from Farm B were 0.00 log MPN/100ml in the borehole source water, while the reservoir dam and irrigation pivot point *E. coli* counts ranged between 0.61-4.56 log MPN/100ml, and 0.00-0.72 log MPN/100ml, respectively (Figure 5.3). Similar to the Enterobacteriaceae and coliform counts, the *E. coli* counts from water samples were significantly different (p<0.0001) (Appendix D Table D4). During the second sampling trip, the reservoir dam water of Farm B had unacceptable *E. coli* levels according to the national regulation for irrigation water (DWAF, 1996b). However, the *E. coli* levels measured during the same trip at the irrigation pivot point in the field was significantly lower with acceptable levels according to the guidelines (Appendix D Table D4). *Escherichia coli* counts of the spinach samples from harvest up to the retailer ranged between 0.00-2.00 log CFU/g (Figure 5.3), and were not significantly different (p=0.7069) (Appendix D Table D5).

The Enterobacteriaceae levels from Farm C ranged between 2.41-3.23 log CFU/ml and 0.00-1.71 log CFU/100ml in the borehole source and irrigation water samples, respectively (Figure 5.4). Enterobacteriaceae counts per trip were significantly lower (p<0.0001) in the irrigation pivot point water compared to the initial borehole source water (Appendix D Table D6). The Enterobacteriaceae levels on spinach from Farm C ranged from 0.00-7.07 log CFU/g (Figure 5.4), with significant differences in the trip x source interactions (p<0.0001) (Appendix D Table D7). Additionally, the Trip 1 unwashed retailer spinach Enterobacteriaceae levels were significantly higher, in comparison to the harvested, after wash, after pack and washed RTE retailed spinach samples (p=0.0042) (Appendix D Table D7). During Trip 2, the Enterobacteriaceae levels from retailed RTE spinach were significantly lower than that of harvested spinach (Appendix D Table D7).

Coliform counts in the irrigation water from Farm C ranged between 4.44-5.44 log MPN/100ml and 0.93-2.44 log MPN/100ml in the borehole source and irrigation pivot point water samples, respectively (Figure 5.4). Although the trip x source water coliform count interactions on Farm C were not significantly different (p=0.0804), the coliform levels from samples from the sources had a significant difference (p<0.0001) with counts from the irrigation pivot point water significantly lower than that of the source water in the dam (Appendix D Table D6). Additionally, coliform count interactions between the two trips were significantly different (p=0.0166) (Appendix D Table D6), with higher levels in Trip 1. The coliform counts on spinach from Farm C ranged between 1.04-7.01 log CFU/g (Figure 5.4) and had significant differences (p<0.0001) (Appendix D Table D7). Similar to the Enterobacteriaceae levels, the Trip 1 unwashed retailer spinach coliform levels were significantly higher, in comparison to the harvested, after wash, after pack and washed RTE retailed spinach samples (p=0.0006) (Appendix D Table D7). Additionally, the Trip 2 washed RTE retailer spinach samples had

significantly higher coliform levels than that of the harvested, packhouse receival, after wash, and after pack samples (p=0.0006) (Appendix D Table D7).

On Farm C, *E. coli* was enumerated in low levels during Trip 1 from the source dam water (borehole) only, with counts ranging between 0.00-0.61 log MPN/100ml. The *E. coli* from the water samples were significantly different (p=0.0014) (Supplementary Table S6), with water from the source dam being significantly higher during Trip 1. *Escherichia coli* counts on spinach from Farm C ranged between 0.00-3.70 log CFU/g (Figure 5.4), with no significant difference (p=0.6166) in *E. coli* levels on spinach from harvest up to retail (Appendix D Table D7).

In the second production scenario, swab samples were taken from the cutting surfaces of the packhouse on Farm C and coliform levels ranged between 0.00-4.93 log CFU/cm^2. Between the two trips, coliform levels were significantly different (p=0.045), with Trip 1 having higher coliform counts (Appendix D Table D8). No *E. coli* was enumerated from the contact surfaces. Similar to the coliform levels, the Enterobacteriaceae levels from the cutting surface swab samples differed significantly (p=0.0333) between the two trips (Appendix D Table D8).

5.3.2 Detection of foodborne pathogens

Overall, 65/288 samples (22.57%) contained *E. coli* after enrichment. A higher number of *E. coli* isolates were recovered from the second production scenario after enrichment, yet the enumerated *E. coli* levels was higher from the first production scenario. *Escherichia coli* isolates (n=80) were recovered from the two spinach production scenarios. This included 35 isolates from the first production scenario from soil (n=1), water (n=13), fresh produce (n=14), and contact surfaces (n=7), whilst the 45 *E. coli* isolates recovered from the second production scenario were from water (n=29) and fresh produce (n=16). Only one *E. coli* isolate from the holding dam water in the first production scenario, was positive for the *stx2* virulence gene,

whilst none of the other diarrheagenic virulence genes tested for were detected. *Salmonella* spp. isolates (n=11) were recovered from two river, one holding dam and one irrigation water samples from the first production scenario. No *Listeria* spp. were isolated from any of the samples.

5.3.4 Phenotypic antimicrobial resistance profiling of Escherichia coli isolates

Of the 80 *E. coli* isolates recovered, 95.00% were resistant against at least one antibiotic. This included resistance to aminoglycosides (73.42%), cephalosporins (50.62%), penicillins (44.30%), tetracyline (37.98%), sulfonamides (21.52%), chloramphenicol (15.19%) and carbapenems (5.06%). Overall, a greater percentage of resistance phenotypes were from water *E. coli* isolates (52.50%), followed by isolates from spinach (37.50%) and contact surfaces (10.00 %) (Figure 5.5 and Figure 5.6) In total, 35/80 (43.75%) of the isolates were multidrug resistant; 26.30% from production scenario one, and 17.50% from the second production scenario, where borehole water was used for irrigation (Table 5.3). The multidrug resistant *E. coli* isolates predominantly showed, within the β-lactam group, resistance to penicillins (66.3%), followed by 4th generation cephalosporins (61.3%) and carbapenems (11.3%). Multidrug resistant phenotypes predominantly included resistance profiles of β-lactams combined with aminoglycosides, followed by β-lactams combined with tetracyclines, sulfonomides, and chloramphenicol, respectively (Table 5.3).

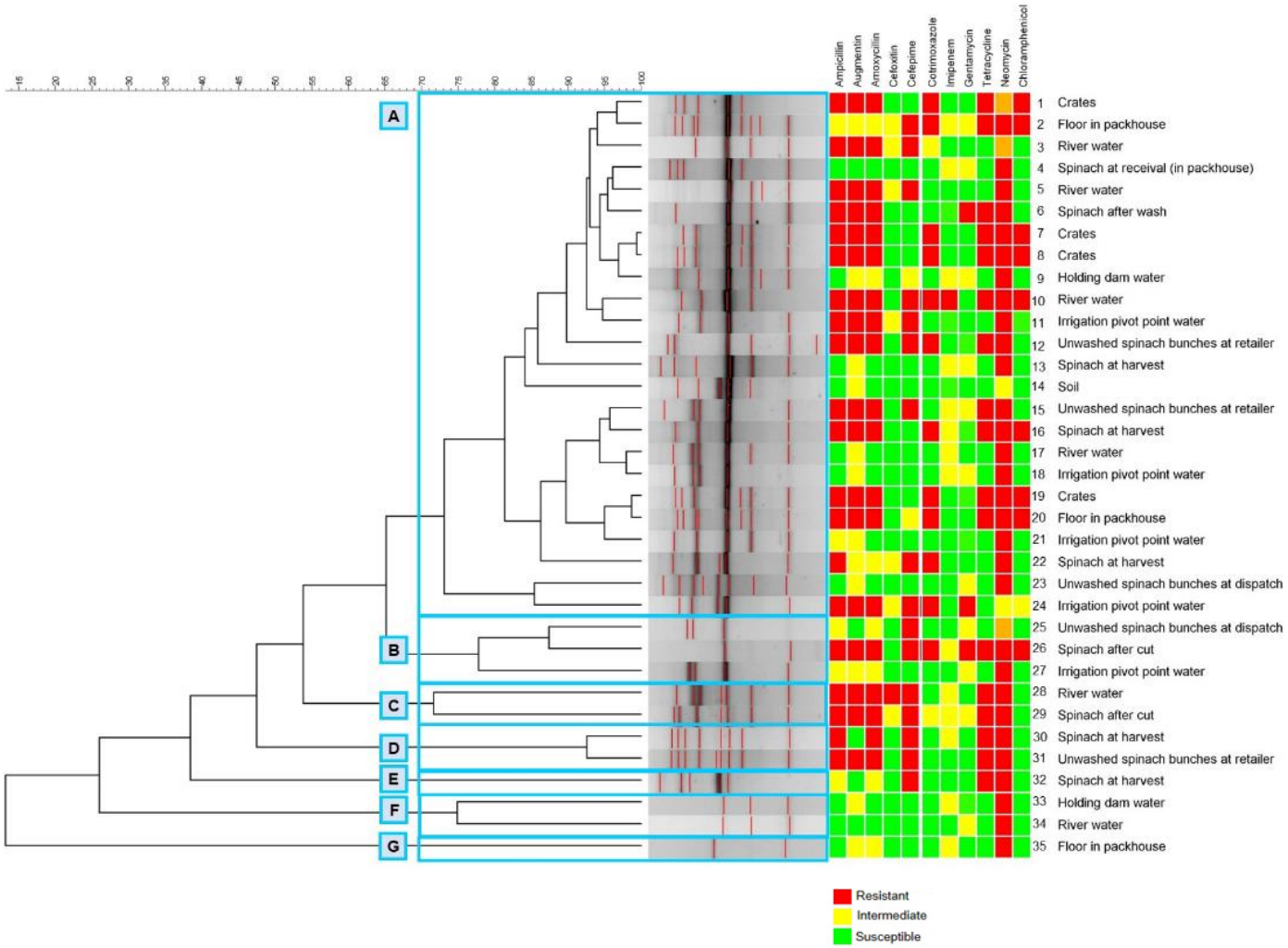

Figure 5.5: Dendrogram showing the genetic relatedness of *Escherichia coli* isolates from irrigation water sources (river, holding dam, and irrigation pivot point), soil, spinach (at harvest, throughout processing and at retail) and contact surfaces throughout spinach production.

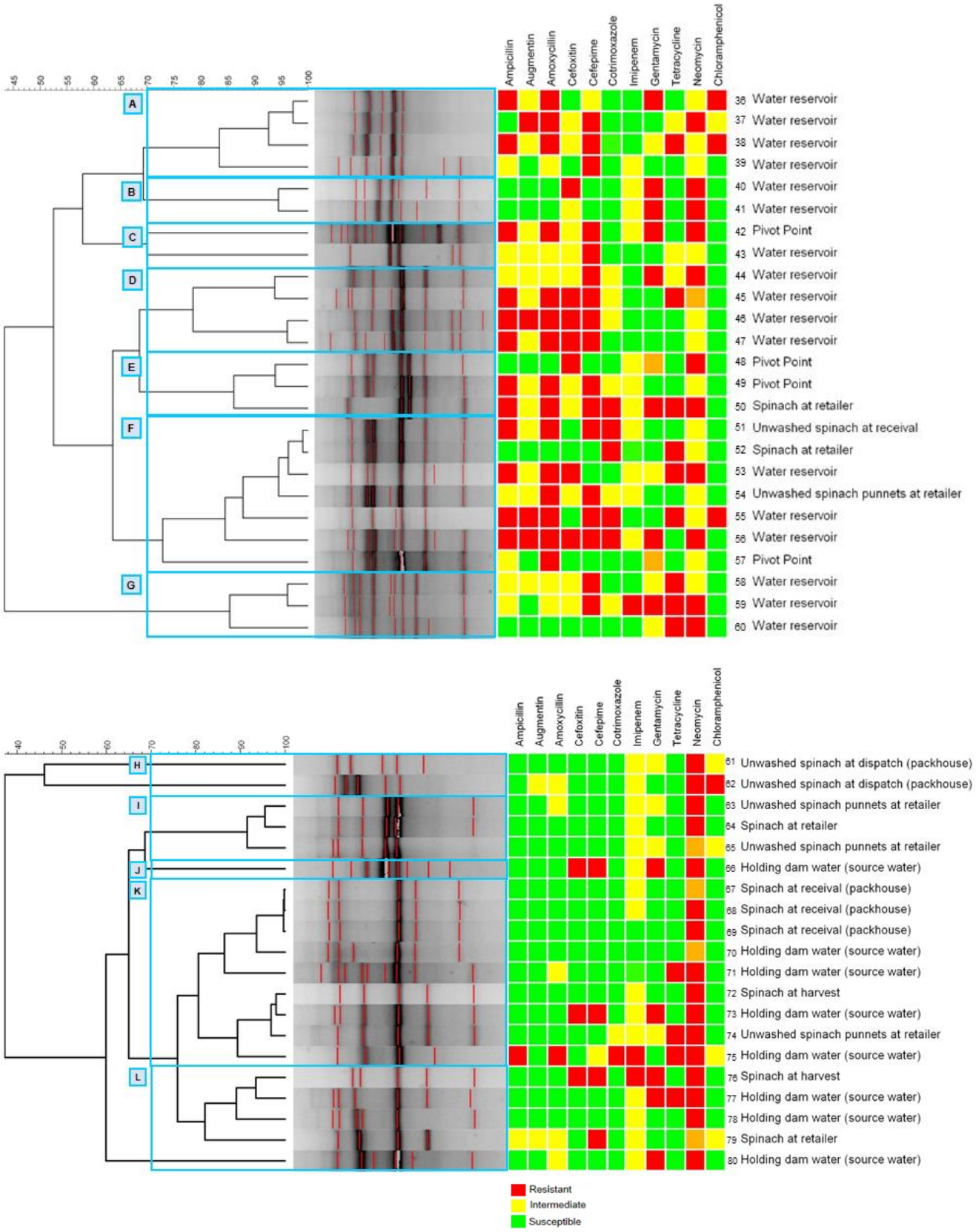

Figure 5.6: Dendrogram showing the genetic relatedness of *Escherichia coli* isolates from irrigation water sources (borehole water sources) and spinach (at harvest, throughout processing and at retail) from two farms supplying spinach to a central processing facility.

Table 5.3: Summary of the number of antimicrobials, most frequent resistance patterns, number, and type of antibiotic classes to which generic *Escherichia coli* isolates from different spinach production scenarios were resistant

No of antimicrobials to which isolates were resistant	No of isolates (n=79)	No of isolates per production scenario		No of isolates with specific pattern	Most frequent pattern[a]	No of antibiotic classes to which isolates were resistant	Antibiotic class(es)
		Production scenario 1	Production scenario 2				
0	4	1	3	4			
		11	6	17	NE10C	1	Aminoglycosides
1	22	1	3	4	CPM30C	1	Cephalosporins
			1	1	A10C	1	Penicillins
			2	2	GM10C - NE10C	1	Aminoglycosides
			3	3	T30C - NE10C	2	Tetracyclines, Aminoglycosides
			1	1	NE10C - C30C	2	Aminoglycosides, Chloramphenicol
2	10		1	1	FOX30C - NE10C	2	Cephalosporins, Aminoglycosides
			1	1	CPM30C - T30C	2	Cephalosporins, Tetracyclines
			1	1	A10C - CPM30C	2	Penicillins, Cephalosporins
			1	1	TS25C - T30C	2	Sulfonomides, Tetracyclines
			1	1	FOX30C - GM10C - NE10C	2	Cephalosporins, Aminoglycosides
			1	1	CPM30C - GM10C - NE10C	2	Cephalosporins, Aminoglycosides
3	5		1	1	GM10C - T30C - NE10C	2	Aminoglycosides, Tetracyclines
			1	1	AP10C - A10C - CPM30C	2	Penicillins, Cephalosporins
		1		1	CPM30C - T30C - NE10C	3	Cephalosporins, Tetracyclines, Aminoglycosides
			2	2	FOX30C - CPM30C - GM10C - NE10C	2	Cephalosporins, Aminoglycosides
		1		1	AP10C - AUG30C - A10C - CPM30C	2	Penicillins, Cephalosporins
			1	1	AP10C - A10C - GM10C - C30C	3	Penicillins, Aminoglycosides, Chloramphenicol
4	8		1	1	AUG30C - A10C - CPM30C - NE10C	3	Penicillins, Cephalosporins, Aminoglycosides
			1	1	AP10C - A10C - FOX30C - CPM30C	2	Penicillins, Cephalosporins
			1	1	AP10C - A10C - CPM30C - TS25C	3	Penicillins, Cephalosporins, Sulfonomides
		1		1	AP10C - CPM30C - TS25C - NE10C	4	Penicillins, Cephalosporins, Sulfonomides, Aminoglycosides
			1	1	AP10C - AUG30C - A10C - FOX30C - CPM30C	2	Penicillins, Cephalosporins
		2		2	AP10C - AUG30C - A10C - CPM30C - NE10C	3	Penicillins, Cephalosporins, Aminoglycosides
			1	1	AP10C - A10C - CPM30C - GM10C - NE10C	3	Penicillins, Cephalosporins, Aminoglycosides
			1	1	FOX30C - CPM30C - IMI10C - GM10C - NE10C	3	Cephalosporins, Carbapenems, Aminoglycosides
			1	1	AP10C - A10C - FOX30C - CPM30C - T30C	3	Penicillins, Cephalosporins, Tetracyclines
5	11	1		1	AP10C - A10C - CPM30C - T30C - NE10C	4	Penicillins, Cephalosporins, Tetracyclines, Aminoglycosides
			1	1	AP10C - A10C - CPM30C - T30C - C30C	4	Penicillins, Cephalosporins, Tetracyclines, Chloramphenicol
			1	1	AP10C - A10C - FOX30C - T30C - NE10C	4	Penicillins, Cephalosporins, Tetracyclines, Aminoglycosides
			1	1	CPM30C - IMI10C - GM10C - T30C - NE10C	4	Cephalosporins, Carbapenems, Aminoglycosides, Tetracyclines
		1		1	CPM30C - TS25C - T30C - NE10C - C30C	5	Cephalosporins, Sulfonomides, Tetracyclines, Aminoglycosides, Chloramphenicol
6	7	1		1	AP10C - AUG30C - A10C - GM10C - T30C - NE10C	3	Penicillins, Aminoglycosides, Tetracyclines

		3		3	AP10C - AUG30C - A10C - CPM30C - T30C - NE10C	4	Penicillins, Cephalosporins, Tetracyclines, Aminoglycosides
		1		1	AP10C - AUG30C - A10C - TS25C - T30C - C30C	4	Penicillins, Sulfonamides, Tetracyclines, Chloramphenicol
		1		1	AP10C - AUG30C - A10C - CPM30C - TS25C - GM10C	4	Penicillins, Cephalosporins, Sulfonomides, Aminoglycosides
			1	1	AP10C - A10C - TS25C - IMI10C - T30C - NE10C	5	Penicillins, Sulfonamides, Carbapenems, Tetracyclines, Aminoglycosides
		1		1	AP10C - AUG30C - A10C - FOX30C - CPM30C - T30C - NE10C	4	Penicillins, Cephalosporins, Tetracyclines, Aminoglycosides
		5		5	AP10C - AUG30C - A10C - TS25C - T30C - NE10C - C30C	5	Penicillins, Sulfonamides, Tetracyclines, Aminoglycosides, Chloramphenicol
7	9	1		1	AP10C - AUG30C - A10C - CPM30C - TS25C - T30C - NE10C	5	Penicillins, Cephalosporins, Sulfonamides, Tetracyclines, Aminoglycosides
			1	1	AP10C - A10C - CPM30C - TS25C - GM10C - T30C - NE10C	5	Penicillins, Cephalosporins, Sulfonamides, Aminoglycosides, Tetracyclines
			1	1	AP10C - AUG30C - A10C - CPM30C - TS25C - T30C - C30C	5	Penicillins, Cephalosporins, Sulfonamides, Tetracyclines, Chloramphenicol
8	1		1	1	AP10C - AUG30C - A10C - FOX30C - CPM30C - TS25C - GM10C - NE10C	4	Penicillins, Cephalosporins, Sulfonamides, Aminoglycosides
		1		1	AP10C - AUG30C - A10C - CPM30C - TS25C - GM10C - T30C - NE10C - C30C	6	Penicillins, Cephalosporins, Sulfonamides, Aminoglycosides, Tetracyclines, Chloramphenicol
9	2	1		1	AP10C - AUG30C - A10C - CPM30C - TS25C - IMI10C - T30C - NE10C - C30C	7	Penicillins, Cephalosporins, Sulfonamides, Carbapenems, Tetracyclines, Aminoglycosides, Chloramphenicol

5.3.5 Enterobacterial Repetitive Intergenic Consensus (ERIC)–PCR cluster analysis and antimicrobial resistance profiles of *Escherichia coli* isolates

At a 70% similarity cut-off, cluster analysis of ERIC-PCR DNA fingerprints generated 7 distinct *E. coli* profiles for the 35 isolates from the first production scenario (Figure 5 A-G). The largest cluster (Cluster A) included *E. coli* isolates (n=24) from water, soil, spinach from farm to retail, as well as contact surfaces through processing. Several water and contact surface samples, as well as spinach at different points throughout production and irrigation water samples clustered together in cluster A with ≥94.0% similarity values. Cluster B included isolates from spinach at different points in the packhouse and irrigation water with similarity values of 78.0%. Similarly, cluster C included an *E. coli* isolate from spinach after cut that was 72.0% similar to a river water isolate. Cluster D was composed of two *E. coli* isolates from spinach (at harvest and at retail) at similarity values >90.0%, whilst in cluster F, two *E. coli* isolates from the river and holding dam water clustered together at 75.0% similarity. Cluster G consisted of a single *E. coli* isolate from the floor swab samples. The *E. coli* ERIC-PCR DNA fingerprints in the second production scenario generated 12 distinct clusters. This included seven clusters in the supply chain from the first supplier, Farm B (Figure 5.6 A-G) and five clusters in the supply chain from the second supplier, Farm C (Figure 5.6 H-L). Cluster E was composed of three *E. coli* isolates from the irrigation pivot point and spinach at retailer, with 86.0% similarity values. In cluster F, several *E. coli* isolates from the water reservoir, spinach at receival in the packhouse as well as washed and unwashed retail spinach clustered together at similarity values ranging from 73.0-99.0%. In cluster I, three *E. coli* isolates from the washed and unwashed spinach product lines at the retailer clustered together with 92.0% similarity. Clusters K consisted of nine *E. coli* isolates, including three spinach at receival isolates and one holding dam isolate with 94.0% similarity. Furthermore, *E. coli* isolates from spinach at harvest, holding dam (source water) and the unwashed spinach at retailer had 98.0% similarity.

The five isolates in cluster L included three *E. coli* isolates from spinach at harvest, and holding dam (source) water with 90.0% similarity.

5.4 Discussion

To the authors knowledge, this is the first study in SA where the spinach supply chain has been studied focusing on different irrigation water sources and the presence of multidrug resistant foodborne pathogens and quality indicator organisms. Higher mean total coliform levels were found in river water than previously reported in similar situations (Jongman and Korsten, 2016). Additionally, total coliforms were detected at enumerable levels in borehole water, in contrast to Jongman and Korsten (2016) where no coliforms were detected in similar sources. As water is central in fresh produce production and processing, and applied in large volumes, it is crucial that the microbiological quality is acceptable (FAO and WHO, 2019; Makinde et al., 2020). Inconsistencies of irrigation water sources quality may impact on the safety of the produce. When spinach was irrigated directly with river water via overhead irrigation as in this study, *E. coli* was found in the irrigation water, spinach, contact surface and wash water samples throughout the supply chain. The average river water *E. coli* levels (2.4 log MPN/100 ml) were similar to the results reported for river water used for overhead irrigation of commercially produced leafy greens in a previous study in Gauteng Province (2.9 log MPN/100 ml) (Jongman and Korsten, 2016). In contrast, *E. coli* was not enumerated from the river water used to irrigate produce in KwaZulu Natal, South Africa (Mdluli et al., 2013). According to the SA Department of Water Affairs and Forestry (DWAF) guidelines of <1000 *E. coli* /100 ml for irrigation water (DWAF, 1996), the river water *E. coli* levels in the current study would have been satisfactory. This is also in agreement with World Health Organisation (WHO) recommendation of <1000 CFU faecal coliforms/100 ml in irrigation water used for minimally processed fresh produce (WHO, 2006). However, the river water *E. coli* levels exceeded the Canadian standards' acceptable limit of <100 *E. coli*/100 ml for irrigation water used for

produce to be consumed raw (Canadian Council of Ministers of the Environment [CCME], 2003). Where borehole water was used for irrigation, the source water *E. coli* levels from the first supplier farm (Farm B) met the current SA and WHO irrigation water standards of <1000 *E. coli* /100 ml (DWAF, 1996; WHO, 2006). *Escherichia coli* levels in the holding dam water did not meet this requirement, reiterating that water quality may affect the microbiological quality of irrigated produce. The *E. coli* levels in the source water from the second supplier farm in production scenario two was acceptable according to the national regulation limits (DWAF, 1996) as well as the Canadian standards' acceptable limit (CCME, 2003).

Internationally, guidelines and regulations for agricultural water quality vary by country/region with different acceptable *E. coli* limits stipulated based on the risk of types of agricultural water systems and specific uses within production and processing (Banach and Van Der Fels-Klerx, 2020; FAO and WHO, 2019). The wash water during processing from the current study had acceptable *E. coli* levels according to the Australia and New Zealand Fresh Produce Safety Centres' guidelines of *E. coli* <100 CFU/ml in pre-wash water to remove soil and debris and *E. coli* <1 CFU/100ml in water for the final wash step of produce that may be eaten uncooked [Fresh Produce Safety Centre Australia & New Zealand (FPSC A-NZ), 2019].

The microbiological characteristics of raw fruit and vegetables are one of the most important properties related to safe fresh produce consumption (Faour-Klingbeil et al., 2016; FAO and WHO, 2019; Schuh et al., 2020). Internationally, no consensus exists regarding the microbiological standards that apply to RTE/ minimally processed vegetables (Health Protection Agency, 2009; [Food Safety Authority of Ireland (FSAI), 2016]; FPSC A-NZ, 2019). A number of countries do suggest exclusion of coliform counts, as high levels are expected due to the natural occurrence (New South Wales Food Authority, 2007; Health Canada, 2010; Centre for Food Safety [CFS], 2014). In SA, the Department of Health (DoH) guidelines stipulated that coliform levels of < 2.3 log CFU/g was acceptable on fresh vegetables

(DoH, 2000), however, these guidelines are currently under revision. Coliforms were enumerated from 98% of the spinach samples in the current study with levels that exceeded 2.3 log CFU/g, similar to other South African studies that reported coliform levels > 2.3 log CFU/g on retailed leafy green vegetables (du Plessis et al., 2017; Richter et al., 2021). Globally, high coliform levels in retailed leafy greens have also been reported (Cerna-Cortes et al., 2015; Korir et al., 2016; Maffei et al., 2016). In contrast to the coliforms, *E. coli* was only enumerated from 8.33% of the spinach samples, thus, 91.6% of the spinach samples had acceptable *E. coli* levels according to the previous DoH *E. coli* guidelines of zero CFU/g (DoH, 2000).

The natural occurrence of Enterobacteriaceae on spinach at various stages of production and processing, regardless of the source of irrigation water, were expected. In the current study, Enterobacteriaceae levels on packed, washed retail spinach samples ranged between 3.56 and 6.52 log CFU/g and on unwashed retail spinach samples between 3.92 and 6.78 log CFU/g. Similar Enterobacteriaceae levels were reported on minimally processed and unprocessed vegetables in Italy, suggesting that the microbial flora can be primarily attributed to a natural environmental source (Cardamone et al., 2015). However, higher Enterobacteriaceae loads could also represent higher loads of potential pathogens such as *E. coli* and *Salmonella* spp. and opportunistic pathogens including *Klebsiella pneumoniae* and *Enterobacter* species (Kilonzo-Nthenge et al., 2018).

After enrichment, generic *E. coli* was isolated from 40.30% and 14.60% of water and spinach samples, respectively. This was lower than the 84.80% and 38.30% generic *E. coli* prevalence in irrigation water and lettuce samples previously reported in Brazil (Decol et al., 2017). Similar to Du Plessis et al. (2015) and Decol et al. (2017), more irrigation water samples in the current study were contaminated with *E. coli* than fresh produce samples. Additionally, only one water *E. coli* isolate was positive for the *stx2* virulence gene. This corresponds to previous

South African studies where a low incidence of virulence genes in *E. coli* from retailed fresh produce were seen (Jongman and Korsten, 2016a; du Plessis et al., 2017; Richter et al., 2021).

Knowledge of the antimicrobial resistance patterns, especially in potential foodborne pathogenic bacteria found throughout fresh produce production systems, is crucial to be able to reduce the number of treatment failures if a foodborne disease outbreak do occur (Kim et al., 2019). In this study, 95% *E. coli* isolates were resistant to at least one antibiotic with 43.75% multidrug resistant. *Escherichia coli* isolates from both irrigation water and spinach in the current study were resistant to antibiotics that are traditionally first-line drug treatment options for gastrointestinal infections (tetracycline, ampicillin and cotrimoxazole) (Alanazi et al., 2018; Kim et al., 2019). More antibiotic resistant *E. coli* isolates were detected from irrigation water (52.5%) than from spinach (37.5%) in the current study, which is similar to antibiotic resistant *E. coli* isolates reported in irrigation water and harvested spinach by Vital et al., (2018). The highest resistance in irrigation water *E. coli* isolates from the current study was against aminoglycosides (35.0%), followed by cephalosporins (28.8%), penicillins (23.8%) and tetracycline (15.0%). In contrast, Vital et al. (2018) reported the highest resistance in *E. coli* isolates from irrigation water in the Philippines against tetracycline (45.6%) and ampicillin (34%).

The ERIC-PCR profiles showed high similarity values (>90.0 %) for irrigation water and spinach *E. coli* isolates at different points of production, processing or retail of each of the respective supply chains. Previous studies have reported the transfer of potential pathogenic enteric bacteria onto produce via irrigation with polluted water (Ijabadeniyi, 2012; Du Plessis et al., 2015). For example, Du Plessis et al. (2015) highlighted the link between irrigation water quality and microbiological quality of onions, whilst Jongman and Korsten (2016a) showed a link between *E. coli* isolates from different leafy green vegetables and the associated irrigation water. Interestingly, cluster analysis within each spinach supply chain in the current study

(regardless of the water source and overall microbiological quality of the irrigation water) showed irrigation water *E. coli* isolates clustering together with *E. coli* from washed and unwashed spinach samples at retail at similarity of at least 85.0%. This indicates that contamination that occurs on the farm can influence the safety of the final product at retail, regardless of processing steps (which often include washing in potable water) followed through production. The importance of irrigation water as contamination source of vegetables, in accordance to previous studies (Du Plessis et al., 2015; Jongman and Korsten, 2016b; Decol et al., 2017), is further reiterated.

5.5 Conclusion

The microbiological quality (Enterobacteriaceae, coliforms and *Escherichia coli*) and prevalence of foodborne pathogens (*E. coli*, *Salmonella* spp. and *Listeria monocytogenes*) including phenotypic (antibiotic resistance) and genotypic (diarrheagenic gene screening and repetitive PCR) characterisation of isolated *E. coli* in two commercial spinach production systems on farm, through processing and up to retail was determined. More antibiotic resistant *E. coli* isolates were detected from irrigation water than from spinach and isolates from irrigation water and spinach at different points of production, processing or retail in each of the respective supply chains had high similarity values. The results from this study provide valuable background information regarding the presence of multidrug resistant environmental *E. coli* throughout spinach production from farm, during processing and up to retail. As antimicrobial resistance is a worldwide public health concern, surveillance of environmental bacteria as possible reservoirs in the water-plant-food interface becomes important. Furthermore, the necessity of using clean and safe irrigation water was highlighted with the need for standardised risk-based microbiological safety parameters for irrigation water of RTE fresh vegetables. This follows as a link between *E. coli* from irrigation water and spinach at different points of the respective production systems were shown. Future work should focus

on determining the presence of ESBL/AmpC-producing Enterobacteriaceae in complete spinach supply chains from farm, through processing and up to retail and to characterise the isolated strains phenotypically and genotypically. This will be addressed in Chapter 6.

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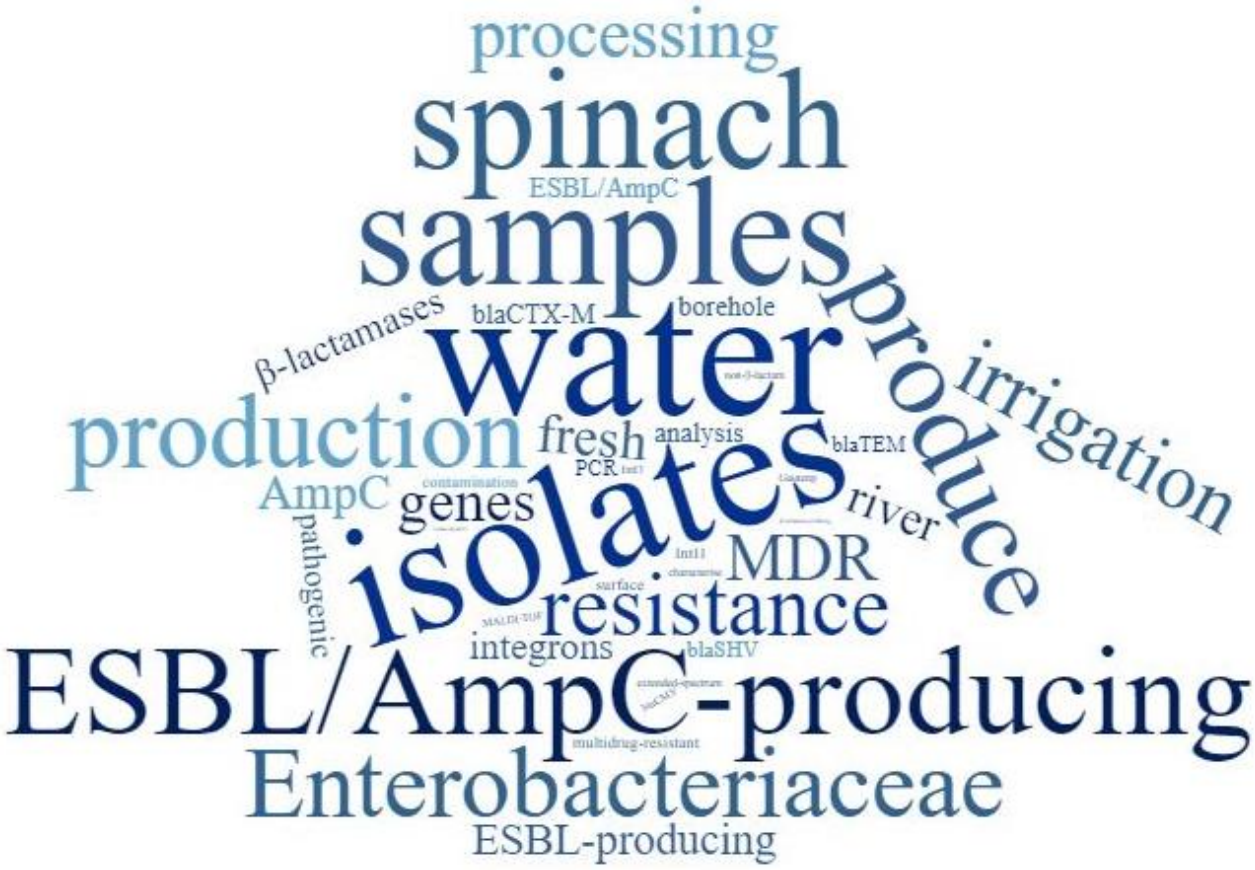
Chapter 6

"We cannot solve problems with the same thinking we used to create them." -*Albert Einstein*



Commercial Spinach Supply Chains

Farm Processing Retail

288 Samples

Characterisation of **E**xtended-**S**pectrum and AmpC **β**-**L**actamase-producing Enterobacteriaceae

14.58 % (42/288) samples were contaminated with ESBL/AmpC producing Enterobacteriaceae

- ESBL-prodocing Enterobacteriaceae isolated from
  15.28 % (11/72) water
  12.12 % (16/132) harvested- and processed spinach
  25 % (15/60) retail spinach samples
- Dominant species:
  Serratia fonticola (45.86 %)
  Escherichia coli (20.83 %)
  Klebsiella pneumoniae (18.75 %)

Presence of ESBL-producing Enterobacteriaceae in selected fresh produce supply chains- persistence of resistance genes on fresh produce throughout processing in different production systems.

- 48 (81.36 %) isolates phenotypically confirmed as ESBL/AmpC-producing Enterobacteriaceae.
- 98 % multidrug resistant.
- CTX-M Group 1 ESBL type dominant, followed by TEM and SHV.

Results highlights the neccesity of surveillance of antimicrobial resistance in different environmental settings

First report on the prevalence of ESBL/AmpC-producing Enterobacteriaceae isolated throughout complete commercial spinach production systems

Richter, L., du Plessis, E. M., Duvenage, S., and Korsten, L. (2020). Occurrence, Phenotypic and Molecular Characterization of Extended-Spectrum- and AmpC- β-Lactamase Producing Enterobacteriaceae Isolated From Selected Commercial Spinach Supply Chains in South Africa. Front. Microbiol. 11, 1–10. doi:10.3389/fmicb.2020.00630.

Plant to Food

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Occurrence, phenotypic and molecular characterization of extended-spectrum- and ampc- β-lactamase producing enterobacteriaceae isolated from selected commercial spinach supply chains in South Africa[6]

Abstract

The increasing occurrence of multidrug-resistant extended-spectrum β-lactamase- (ESBL) and/or AmpC β-lactamase-producing Enterobacteriaceae in health care systems, the environment and fresh produce is a serious concern globally. Production practices, processing and subsequent consumption of contaminated raw fruit and vegetables represent a possible human transmission route. The purpose of this chapter was to determine the presence of ESBL/AmpC-producing Enterobacteriaceae in complete spinach supply chains and to characterise the isolated strains phenotypically (antimicrobial resistance profiles) and genotypically (ESBL/AmpC genetic determinants, detection of class 1, 2, and 3 integrons). Water, soil, fresh produce and contact surface samples (n=288) from two commercial spinach production systems were screened for ESBL/AmpC-producing Enterobacteriaceae. In total, 14.58 % (42/288) of the samples were found to be contaminated after selective enrichment, plating onto chromogenic media and matrix-assisted laser desorption ionization time-of-flight mass spectrometry identity confirmation of presumptive ESBL/AmpC isolates. This included 15.28 % (11/72) water and 12.12 % (16/132) harvested- and processed spinach, while 25 % (15/60) retail spinach samples were found to be contaminated with an increase in isolate abundance and diversity in both scenarios. Dominant species identified included *Serratia fonticola* (45.86 %), *Escherichia coli* (20.83 %), and *Klebsiella pneumoniae* (18.75 %). In total, 48 (81.36 %) isolates were phenotypically confirmed as ESBL/AmpC-producing Enterobacteriaceae of which 98 % showed a multidrug-resistant phenotype. Genotypic

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characterisation (PCR of ESBL/AmpC resistance genes and integrons) further revealed the domination of the CTX-M Group 1 ESBL type, followed by TEM and SHV; whilst the CIT-type was the only plasmid-mediated AmpC genetic determinant detected. Integrons were detected in 79.17 % (n=38) of the confirmed ESBL/AmpC-producing isolates, of which we highlight the high prevalence of class 3 integrons, detected in 72.92 % (n=35) of the isolates, mostly in *S. fonticola*. Class 2 integrons were not detected in this study. This is the first report on the prevalence of ESBL/AmpC-producing Enterobacteriaceae isolated throughout commercial spinach production systems harbouring class 1 and/or class 3 integrons in Gauteng Province, South Africa. The results add to the global knowledge base regarding the prevalence and characteristics of ESBL/AmpC-producing Enterobacteriaceae in fresh vegetables and the agricultural environment required for future risk analysis.

6.1 Introduction

The prevalence of multidrug-resistant (MDR) human pathogenic bacteria and their genetic determinants have increased significantly in clinical and environmental settings due to the overuse of antibiotics (Jones-Dias et al., 2016). Subsequently, treatment options for infections become limited, especially when these MDR pathogens harbour genes expressing resistance to extended spectrum antibiotics (Freitag et al., 2018). Production of β-lactamases, including extended-spectrum- and AmpC β-lactamases is one of the most significant resistance mechanisms among Enterobacteriaceae (Östholm, 2014). Enterobacteriaceae is a large family of Gram-negative bacteria present in water, soil and plants, including fresh vegetables where they form part of the indigenous microbiota (Blaak et al., 2014). The family also includes important foodborne pathogens such as pathogenic *Escherichia coli* and *Salmonella* spp., as well as opportunistic pathogens including *Klebsiella pneumoniae, Serratia*- and *Citrobacte*r spp. (Baylis et al., 2011).

Extended-spectrum β-lactamase (ESBL) and AmpC β-lactamase enzymes are capable of inactivating nearly all β-lactam antibiotics, differing only in their capacity to hydrolyze fourth-generation cephalosporins (Blaak et al., 2014). The ESBLs are classified as Ambler class A enzymes and include TEM-, SHV-, OXA-, and CTX-M enzymes (Bush and Jacoby, 2010). In the 1980s resistance to third-generation cephalosporins were mainly due to the production of TEM and SHV enzymes (Bush and Jacoby, 2010). However, since the early 2000s, production of CTX-M enzymes have predominantly been reported (Bush and Jacoby, 2010; Ye et al., 2017a). AmpC β-lactamases, classified as Ambler class C enzymes, contrast class A enzymes in being active against cephamycins (e.g. cefoxitin) and resistant to inhibition by clavulanic acid (Bush and Jacoby, 2010). Plasmid-mediated AmpC (pAmpC) β-lactamases belong to six families including EBC, CIT, ACC, DHA, FOX and MOX (Bush and Jacoby, 2010).

Fresh produce have increasingly been reported to constitute a reservoir of ESBL/AmpC-producing Enterobacteriaceae and their associated genetic determinants (Blaak et al., 2014; Ye et al., 2017; Freitag et al., 2018; Iseppi et al., 2018). Bacteria can readily acquire genes for production of ESBL/AmpC β-lactamases, with mobile genetic elements (e.g. integrons) aiding the dissemination process (Schill et al., 2017). Three classes of integrons, classified based on the more conserved amino acid sequences of the integrase gene (*IntI*), are known to be associated with antimicrobial resistance genes (Machado et al., 2005; Kargar, et al., 2014; Deng et al., 2015).

Transfer of MDR ESBL/AmpC-producing Enterobacteriaceae onto fresh produce can occur through the use of contaminated irrigation water or during production via animal manure, during processing, transport and at the point-of-sale (van Hoek et al., 2015). In fact, contaminated irrigation water has been identified as a main contributor of antimicrobial

resistance build up in environmental settings (Soodb et al., 2018). Consumption of contaminated raw vegetables can therefore potentially have a negative impact on human health, as antimicrobial resistance genes can be transferred to commensal bacteria which typically colonize the human gut (Ye et al., 2017a). In addition, the WHO has reported that leafy greens in particular represent a higher risk for the consumer (WHO, 2008).

The presence of ESBL/AmpC-producing Enterobacteriaceae on leafy green vegetables at the point of sale have been reported worldwide (Kim et al., 2015; Nüesch-Inderbinen et al., 2015; Usui et al., 2019; Zurfluh et al., 2015). Other studies have evaluated the presence of ESBL-producing Enterobacteriaceae in samples from both retail vegetables and the agricultural environment in Tunisia (Ben Said et al., 2015), China (Ye et al., 2017a) and the Netherlands (Blaak et al., 2014). In South Africa, transfer of extended spectrum and AmpC β-lactamase genetic determinants between antimicrobial resistant *E. coli* strains from irrigation water to lettuce were reported (Njage and Buys, 2014), while a recent study reported a high prevalence of ESBL/AmpC-producing Enterobacteriaceae on spinach samples at retail (Richter et al., 2019). However, no studies have investigated the spread of ESBL/AmpC-producing Enterobacteriaceae and prevalence of integrons that potentially aid in dispersal of these resistance genes throughout the fresh produce supply chains. This include the on farm environment, harvesting, processing and packaging, up to the point of sale. This study aimed to determine the presence of ESBL/AmpC-producing Enterobacteriaceae in typical commercial spinach production systems from the farm to retail, and to characterise the isolated strains by (i) phenotypic antimicrobial resistance profiles, (ii) identification of ESBL/AmpC genetic determinants, and (iii) detection of Class 1,2 and 3 integrons.

6.2 Materials and Methods

6.2.1 Sampling study areas

Samples were collected from two different commercial spinach production scenarios typically seen in vegetables supply chains in Gauteng Province, SA from June to November 2017. The first scenario consisted of a GLOBAL-GAP certified farm (Farm A) that used river water with overhead irrigation and open field cultivation. Depending on the field layout, river water was either used directly or used after storing in a holding dam. The processing facility was located on the farm where spinach was either washed, dried, cut, packed or made up in bunches and sent to national fresh produce markets, retailers and/or retail-distribution centres. The second spinach production scenario used a central processing facility and received produce from various farms. Two GLOBAL-GAP certified farms (Farm B and Farm C, located 112 km and 105 km, respectively, from the processing facility) were selected for sampling of baby spinach. Both farms used borehole water for irrigation and produce were grown in tunnels. On Farm B, borehole water was circulated between two holding dams, while one big holding dam was used on Farm C.

6.2.2 Sample collection and processing

A total number of 288 samples were collected throughout the supply chains from the two spinach production scenarios (Appendix E Figure E1). This included soil at harvest (n=6 composite samples); water samples at the source, irrigation point and during processing (n=72); spinach samples at harvest, during processing and at retail (n=192); and contact surface swab samples throughout production and processing of the fresh produce (n=18).

Soil. Soil was collected from five replicate points during harvest from the spinach production fields. A composite sample of 25g (5g from each replicate) were added to 225ml buffered

peptone water (BPW) and incubated for 3-4 h at 37 °C prior to enrichment for detection and isolation of presumptive ESBL/AmpC-producing Enterobacteriaceae.

Water. From each water sampling point (source-, irrigation pivot point- and wash water), 1 L water samples were collected in triplicate and each sample filtered through a 0.45 µm nitrocellulose membrane (Sartorius, Johannesburg, SA). The membrane was subsequently placed into 50 ml BPW and incubated for 3-4 h at 37 °C prior to enrichment for presumptive ESBL/AmpC-producing Enterobacteriaceae.

Fresh produce. After removal of the spinach stalks, at least three leaves were used to prepare 50 g composite samples. For the baby spinach, 50 g composite samples were obtained. Each sample was aseptically cut and placed into a sterile polyethylene strainer stomacher bag (Seward Ltd., London, UK) containing 200 ml (3M, Johannesburg) BPW in a 1:4 weight to volume ratio. Individual vegetable samples were blended for 5 min at 230 rpm in a Stomacher 400 circulator paddle blender (Seward Ltd., London) and incubated for 3-4 h at 37 °C prior to enrichment for presumptive ESBL/AmpC-producing Enterobacteriaceae.

Contact surfaces. Transystem^{TM} swabs with Amies medium (Lasec, Johannesburg) were used to sample a 25cm^2 area from crates, tables and conveyer belt surfaces respectively, in triplicate, according to the standard procedures for environmental swab sampling (Public Health England, 2014). Swabs were analysed by placing each into 9 ml BPW for the 3-4 h enrichment at 37 °C prior to enrichment for presumptive ESBL/AmpC-producing Enterobacteriaceae.

6.2.3 Isolation and identification of presumptive ESBL/AmpC-producing Enterobacteriaceae

Presumptive ESBL/AmpC-producing Enterobacteriaceae were isolated and identified as previously described (Richter et al., 2019). Briefly, each of the prepared BPW-samples were incubated for 3-4 h at 37 °C after which 1 ml was added to 9 ml Enterobacteriaceae enrichment

(EE) broth (Oxoid, Johannesburg) and incubated overnight at 30 °C. Presumptive ESBL/AmpC-producing microorganisms were detected by streaking (10 µl) each of the enriched samples onto ChromID ESBL agar plates (bioMérieux, Midrand, SA) and incubated overnight at 30 °C (Blaak et al., 2014). All presumptive positive ESBL/AmpC-producing Enterobacteriaceae colonies were isolated and purified. Isolates were identified using matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI- TOF) (Bruker, Bremen, Germany) to species level as described by Standing et al. (2013) and AOAC-OMA#2017.09. Briefly, the purified presumptive positive ESBL/AmpC-producing Enterobacteriaceae colonies were regrown in 9 ml TSB and incubated overnight at 37 °C. Subsequently, isolates were streaked out on nutrient agar (MERCK) and the plates were incubated overnight at 37 °C and colonies formed on the plates were subjected to the MALDI Biotyper protocol (Bruker, Bremen, Germany). All strains were tested in duplicate (Appendix E Table E1). The best organism match score values ranging between 2.300-3.00 were considered reliable for identification at the species level, whilst the best organism match score values ranging between 2.00 -2.299 were considered reliable for genus level, with probable species identification, and values between 1.700-1.999 were considered as probable genus identification.

6.2.4 Antimicrobial susceptibility testing

Antimicrobial susceptibility was tested using the Kirby Bauer disk diffusion technique [Clinical Laboratory Standard Institute (CLSI), 2018]. All isolates were screened for ESBL production by the double-disk synergy test (DDST) using cefotaxime-30 µg, ceftazidime-30 µg, and cefpodoxime-10 µg, alone or in combination with clavulanic acid-10 µg (Mast Diagnostics, Randburg, SA) [European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2013]. To determine if isolates were resistant, intermediate or susceptible, zone

diameters were measured and compared to the CLSI and EUCAST breakpoint criteria. Isolates showing resistance to cefoxitin and cefotaxime or ceftazidime were regarded as a phenotypic indicator of AmpC production (EUCAST, 2013). The cefepime ESBL disc set (Cefepime-30 µg, cefepime-clavulanic acid-30 µg-10 µg) and the AmpC detection set (Mast Diagnostics, Randburg) were used to confirm ESBL and AmpC production, respectively (EUCAST, 2013; CLSI, 2018). Resistance or susceptibility of isolates were also tested using ampicillin-10 µg, augmentin-20 µg/10 µg, amoxicillin-10 µg, cotrimoxazole-1.25µg/23.75 µg, imipenem-10 µg, neomycin-10 µg, tetracycline-30 µg, gentamycin-10 µg, chloramphenicol-10 µg (Mast Diagnostics) (CLSI, 2018). Isolates resistant to three or more antimicrobial classes were regarded MDR. According to the manufacturers' instructions *K. pneumoniae* ATCC 700603, *E. coli* NCTC 13351, and *Enterobacter cloacae* NCTC 1406 were used as positive controls and *E.* ATCC 25922 were included as a negative control (Mast Diagnostics).

6.2.5 Detection of β-lactamase genes and integrons

All confirmed ESBL/AmpC-producing isolates were analysed by PCR and sequencing for the presence of ESBL determinants (*bla*TEM, *bla*SHV, *bla*CTX-M, *bla*OXA) and plasmid-mediated AmpC (pAmpC) resistance genes (*bla*ACC, *bla*FOX, *bla*MOX, *bla*DHA, *bla*CIT, *bla*EBC) as well as class 1, 2, and 3 integrons (*IntI*1, *IntI*2, *IntI*3). Single colonies of each isolate were cultured aerobically under shaking conditions at 200 rpm in tryptone soy broth (TSB) (MERCK, Johannesburg) for 24 h at 30 °C. The cells were pelleted by centrifugation (12,500 *g* for 10 min), DNA was extracted using the Quick-gDNA Mini-Prep kit (Zymo Research, Irvine, USA) and the DNA concentration was determined using the Qubit dsDNA Broad Range Assay and a Qubit 2.0 fluorometer (Life Technologies, Johannesburg). PCR was performed using the DreamTaq Green PCR Master Mix (ThermoFisher Scientific, Johannesburg) with specific primers and thermocycling conditions for each of the genes as

described in Table 6.1. PCR products were sequenced using BigDye Terminator v3.1 cycle sequencing on an ABI 3500XL sequencer in forward and reverse direction (InquabaBiotec, Johannesburg). The sequences were edited with Chromas 2.6 and BioEdit sequence alignment editor software and consensus sequences were subjected to BLAST nucleotide search analysis to identify the antimicrobial resistance genes.

6.3 Results

6.3.1 Isolation and identification of presumptive ESBL/AmpC-producing Enterobacteriaceae isolates

Presumptive ESBL/AmpC-producing Enterobacteriaceae (n=59) from the selective chromogenic media belonged to six genera including *Escherichia*, *Klebsiella*, *Serratia*, *Rahnella*, *Salmonella*, and *Enterobacter*, with MALDI-TOF analysis (Appendix D Table D1). All presumptive ESBL/AmpC-producing Enterobacteriaceae from the selective chromogenic media had best organism match score values >1.700 and <3.00 (Appendix D Table D1). According to the MALDI-TOF score value description, a total of 66.10 % of the isolates were characterised to highly probable species identification, 27.12 % were characterised to secure genus identification and probable species identification, whilst 6.78 % were characterised to probable genus identification (Appendix D Table D1). This included isolates from the water (n=20), fresh produce (n=35) and contact surface samples (n=4), while no presumptive ESBL/AmpC-producing Enterobacteriaceae isolates were recovered from the soil samples.

Table 6.1: Primers used for screening of broad-spectrum β-lactamase, ESBL and AmpC genetic determinants (Dallenne et al., 2010) as well as integron prevalence (de Paula et al., 2018) in selected Enterobacteriaceae isolated from water, fresh produce and contact surfaces

Target genes	Primer sequences	Thermocycling conditions	Expected amplicon size (bp)
bla$_{TEM}$	TEM-F: 5'-CATTTCCGTGTCGCCCTTATTC-3' TEM-R: 5'-CGTTCATCCATAGTTGCCTGAC-3'	94°C, 10min; 30 cycles of 94°C, 40s, 58°C, 40s, 72°C 1min; 72°C 7min	800
bla$_{SHV}$	SHV-F: 5'-AGCCGCTTGAGCAAATTAAAC-3' SHV-R: 5'-ATCCCGCAGATAAATCACCAC-3'		713
bla$_{OXA\text{-}1\ like}$	OXA-F: 5'-GGCACCAGATTCAACTTCAAG-3' OXA-R: 5'-GACCCCAAGTTTCCTGTAAGTG-3'		564
bla$_{CTX\text{-}M\ Group\ 8/25}$	CTX-M Gp8/25-F: 5'-AACRCRCAGACGCTCTAC-3' CTX-M Gp8/25-R: 5'-TCGAGCCGGAASGTGTYAT-3'	94°C, 10min; 30 cycles of 94°C, 40s, 60°C, 40s, 72°C 1min; 72°C 7min	326
bla$_{CTX\text{-}M\ Group\ 9}$	CTX-M Gp9-F: 5'-TCAAGCCTGCCGATCTGGT CTX-M Gp9-R: 5'-TGATTCTCGCCGCTGAAG-3'		688
bla$_{CTX\text{-}M\ Group\ 1}$	CTX-M Gp1-F: 5'-TTAGGAARTGTGCCGCTGYA-3' CTX-M Gp1-R: 5'-CGATATCGTTGGTGGTRCCAT-3'		561
bla$_{ACC}$	ACC-F: 5'-CACCTCCAGCGACTTGTTAC-3' ACC-R: 5'-GTTAGCCAGCATCACGATCC-3'	94°C, 10min; 30 cycles of 94°C, 40s, 60.5°C, 40s, 72°C 1min; 72°C 7min	346
bla$_{FOX}$	FOX-F: 5'-CTACAGTGCGGGTGGTTT-3' FOX-R: 5'-CTATTTGCGGCCAGGTGA-3'	94°C, 10min; 30 cycles of 94°C, 40s, 59.6°C, 40s, 72°C 1min; 72°C 7min	162
bla$_{MOX}$	MOX-F: 5'-GCAACAACGACAATCCATCCT-3' MOX-R: 5'-GGGATAGGCGTAACTCTCCCAA-3'		895
bla$_{DHA}$	DHA-F: 5'-TGATGGCACAGCAGGATATTC-3' DHA-R: 5'-GCTTTGACTCTTTCGGTATTCG-3'		997
bla$_{CIT}$	CIT-F: 5'-CGAAGAGGCAATGACCAGAC-3' CIT-R: 5'-ACGGACAGGGTTAGGATAGY-3'		538
bla$_{EBC}$	EBC-F: 5'-CGGTAAAGCCGATGTTGCG-3' EBC-R: 5'-AGCCTAACCCCTGATACA-3'		683
IntI1	Int1-F: 5'-GGT CAAGGATCTGGATTTCG-3' Int1-R: 5'-ACATGCGTGTAAATCATCGTC-3'	94°C, 12min; 30 cycles of 94°C, 30s, 60°C, 30s, 72°C 1min; 72°C 8min	436
IntI2	Int2-F: 5'-CACGGATATGCGACAAAAAGG-3' Int2-R: 5'-TGTAGCAAACGAGTGACGAAATG-3'		788
IntI3	Int3-F: 5'-AGTGGGTGGCGAATGAGTG-3' Int3-R: 5'-TGTTCTTGTATCGGCAGGTG-3'		600

6.3.2 Prevalence of extended-spectrum β-lactamae and/or AmpC-producing Enterobacteriaceae and antimicrobial susceptibility testing

In total, screening using DDST, 48/59 (81.36 %) isolates tested positive for ESBL production (Figure 6.1). All cefoxitin resistant isolates (20/59) were additionally screened with the AmpC detection set of which 11/20 (55 %) tested positive (Figure 6.1). From the 48 ESBL/AmpC-producing isolates, 16 isolates were from water and 32 from produce samples. Irrigation water isolates (n=15) included *E. coli* (14.58 %) and *Serratia fonticola* (6.25 %) from both scenarios, while *K. pneumoniae* (6.25 %) and *Salmonella* spp. (4.17 %) were isolated only from scenario 1 where river water was used for irrigation. Isolates from the spinach at harvest and throughout processing (n=13) included predominantly *S. fonticola* (16.67 %), followed by *K. pneumoniae* (4.17 %), *Rahnella aquatilis* (4.17 %) and *E. coli* (2.08 %). From the retailer spinach (n=19), ESBL/AmpC-producing *S. fonticola* (16.67 %), *K. pneumoniae* (8.33 %), *R. aquatilis* (6.25 %), *E. coli* (4.17 %), and *Enterobacter asburiae* (2.08 %) were recovered. One *R. aquatilis* isolate was also recovered from the wash water used during processing in scenario 1 (Figure 6.1).

Multidrug resistance was observed in 98 % of the confirmed ESBL/AmpC-producing isolates, including 16 and 31 isolates from water and fresh produce, respectively (Figure 6.1). Resistance to the aminoglycoside (89.58 %) and chloramphenicol (79.17 %) classes were dominant. Within the β-lactam group, further analysis showed resistance against amoxicillin (31.25 % in water and 66.67 % in produce), followed by ampicillin (29.17 % in water and 66.67 % in produce), augmentin (29.17 % in water and 52.08 % in produce), and cefoxitin (14.58 % in water and 27.08 % in produce). The resistance rate to carbapenems (imipenem) were 8.33 % and 4.17 % in water and produce, respectively, with 10.42 % and 41.67 % of the water and produce isolates that showed intermediate resistance to imipenem. Resistance to other antibiotics included cotrimoxazole (22.92 % in water and 29.17 % in produce) and tetracycline (22.92% in water and 27.08 % in produce).

6.3.3 Genotypic antibiotic resistance profiling

Genes encoding β-lactamases were detected in 29/48 (60.42 %) isolates obtained from water and produce samples, mainly in *S. fonticola* (n=13), followed by *E. coli* (n=7) and *K. pneumoniae* (n=5). The most frequently detected β-lactamase genes were *bla*CTX-M (n=25), followed by *bla*TEM (n=18), bla_{SHV} (n=17) and *bla*OXA (n=12). Extended-spectrum β-lactamase variants encoded by *bla*CTX-M Group 1 included CTX-M-3, CTX-M-12, and CTX-M-15 amongst others, whilst *bla*CTX-M Group 9 encoded for CTX-M-14. The bla_{TEM} sequences were found to encode for the broad-spectrum β-lactamase TEM-1 and TEM-234. The bla_{SHV} sequences encoded SHV-187, SHV-203 or SHV-61. All the *bla*OXA sequences encoded broad-spectrum β-lactamases OXA-1. Only the CIT family (identified as bla_{CMY} variants) of AmpC genetic determinants was detected in six *S. fonticola* isolates from scenario 2 (Figure 6.1).

6.3.4 Detection of integrons

The integrase 1 gene (*IntI1*) was detected in 23/48 (47.92 %) of the isolates, predominantly in *S. fonticola* (n=11), followed by *K. pneumoniae* (n=6), *R. aquatilis* (n=2), *E. coli* (n=3), and one *E. asburiae* isolate. The *IntI3* gene associated with class 3 integrons were detected in 35/48 (72.92 %) of the isolates, including *S. fonticola* (n=16), six *E. coli*, six *K. pneumoniae*, five *R. aquatilis*, and one *E. asburiae* and *Salmonella* spp. isolate, respectively. Both the class 1 and class 3 integrase genes were detected in 29 isolates, which included *S. fonticola* (n=9), *K. pneumoniae* (n=5), *E. coli* (n=3), *R. aquatilis* (n=2) and *E. asburiae* (n=1). Class 2 integrons were not detected in any of the isolates (Figure 6.1).

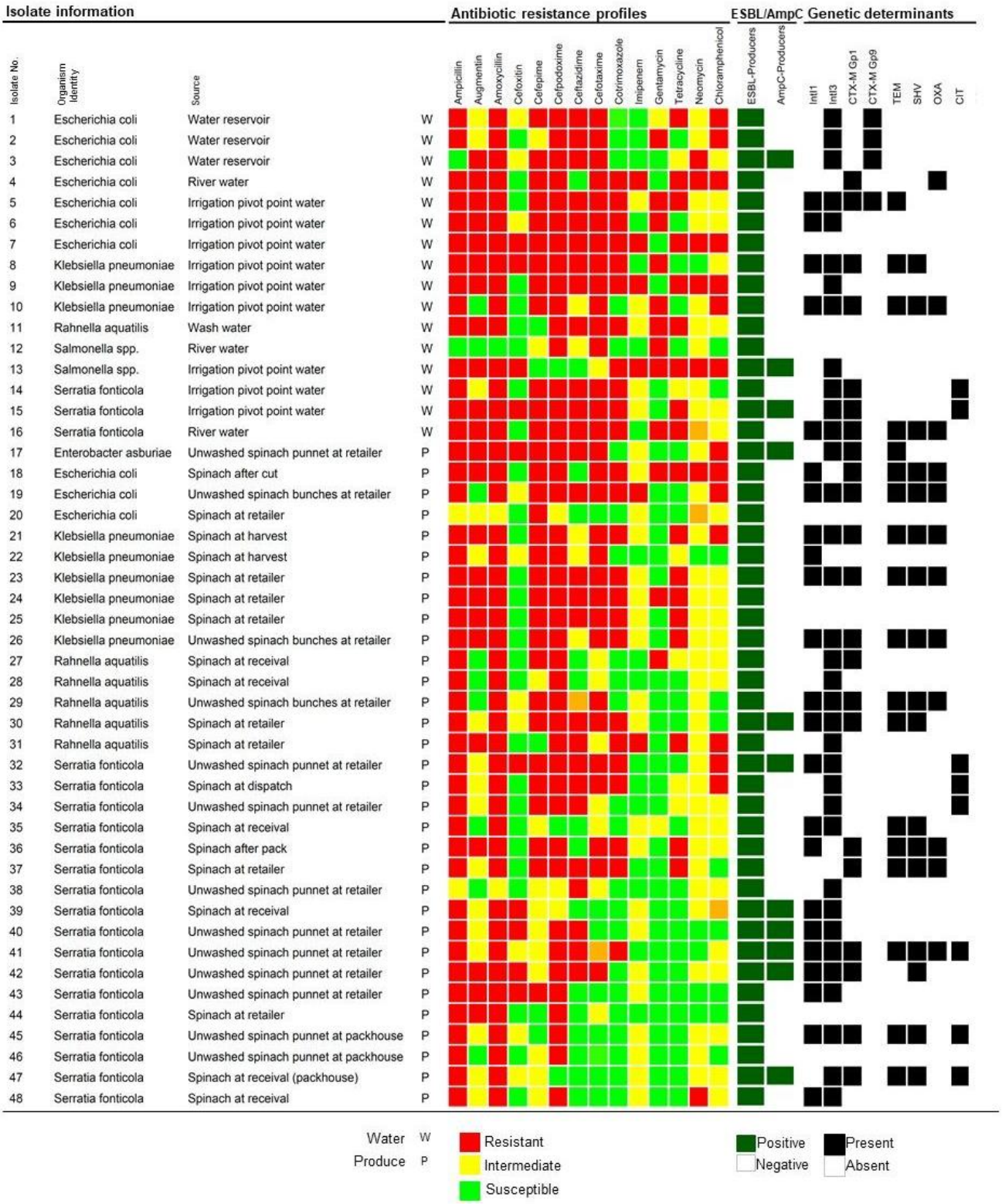

Figure 6.1: Extended-Spectrum- and AmpC- β-Lactamase producing Enterobacteriaceae isolated from water, spinach and contact surface sources, indicating the phenotypic antibiotic resistance profiles and the detection of ESBL and/or AmpC, and integron genetic determinants. The colour code of the antimicrobial resistance profiles indicate the resistant, intermediate resistant or susceptible phenotypes to specific antibiotics from seven different classes. ESBL/AmpC production is indicated as positive or negative and detection of genetic determinants indicated as present or absent.

6.4 Discussion

This study documents the prevalence of ESBL/AmpC-producing Enterobacteriaceae in spinach production, from the agricultural environment, during processing, and subsequent retailed products in SA. Overall, six ESBL/AmpC-producing Enterobacteriaceae genera, including environmental bacteria (*S. fonticola* and *R. aquatilis*), and potential human pathogens (*E. coli, K. pneumoniae*, *Salmonella* spp. and *E. asburiae*) were detected from 42 of the 288 samples. From the first production scenario, ESBL-producing potential pathogenic Enterobacteriaceae were mainly isolated, whereas the predominance of ESBL-producing *S. fonticola* from the second production scenario correspond to environmental ESBL-producing Enterobacteriaceae previously reported (Blaak et al., 2014).

Irrigation water is a known source of antimicrobial resistant bacterial contamination in fresh produce production (Vital et al., 2018; Koutsoumanis et al., 2021). In both spinach production scenarios, the prevalence of ESBL/AmpC-producing Enterobacteriaceae (n=48) was higher in samples from produce (29.17 % and 37.5 %, respectively) than river (20.83 %) and borehole (10.42 %) water. Similarly, Njage and Buys (2014) reported highest prevalence of ESBL-producing *E. coli* isolates in fresh produce (lettuce) at harvest (90 %), followed by different irrigation water (canal, 73 % and river, 64 %) samples in South Africa. In contrast, 100 % irrigation water samples and only 14.7 % of the harvested lettuce samples were found to be positive for ESBL/AmpC-producing environmental Enterobacteriaceae in the Netherlands (Blaak et al., 2014). The 20.83 % (10/48) occurrence of ESBL/AmpC-producing isolates from river irrigation water was higher than the 13.2 % reported in a similar study from river water in China (Ye et al., 2017a). Potential pathogenic ESBL-producing *K. pneumoniae*, *E. coli* and *Salmonella* spp. found in our river water samples were similar to the ESBL-producing potential pathogenic *E. coli*, *Citrobacter freundii* and *K. pneumoniae* reported by Ye et al. (2017). In contrast to Zekar et al. (2017), a 10.4 % occurrence of ESBL/AmpC-producing isolates (*E. coli* and *S. fonticola*) was found in borehole irrigation water from the second production scenario. The occurrence of ESBL/AmpC-producing

Enterobacteriaceae on all our spinach samples increased from 6.25 % at harvest, to 34.38 % after processing, up to 59.36 % in retail spinach samples in both production scenarios. Furthermore, an increase in species diversity from harvested, to processed-, and subsequent retail spinach were also observed. The identified species on retailer spinach samples included ESBL/AmpC-producing *K. pneumoniae*, *S. fonticola*, *R. aquatilis*, *E. coli* and *E. asburiae*, similar to other studies (Ye et al., 2017; Zekar et al., 2017; Richter et al., 2019). Interestingly, no ESBL/AmpC-producing Enterobacteriaceae isolates were detected in soil samples from any of the farms analysed in the current study, which contrasts to Ben Said et al. (2015) and Blaak et al. (2014), where ESBL/AmpC-producing *E. coli* and *S. fonticola* respectively, were detected in soil samples at harvest, respectively.

In this study, 98 % of the ESBL/AmpC-producing isolates were multidrug resistant, while 93.3 % MDR have been reported for ESBL-producing isolates from a similar study in Tunisia (Ben Said et al., 2015). Moreover, 100 % of the river irrigation water isolates from this study showed MDR phenotypes, which is significantly higher than the 42.3 % MDR previously reported in ESBL-producing Enterobacteriaceae isolates from river water (Ye et al., 2017a). Overall, 63.16 % (12/19) of the isolates from retailed spinach showed a MDR phenotype, which is lower than the 83.78 % MDR previously reported on retail spinach in South Africa (Richter et al., 2019). In addition, resistance to as many as four additional non-β-lactam antibiotic classes were observed in the MDR ESBL-producing potential pathogenic isolates from river water and spinach samples. This included *K. pneumoniae* isolates with resistance to cotrimoxazole, a clinically relevant antibiotic, similar to clinical isolates in a recent South African study (Vasaikar et al., 2017). The occurrence (36 %) of MDR ESBL-producing *K. pneumoniae* throughout the first production scenario was high, compared to similar studies where 0 % (the Netherlands) and 15 % (China) occurrence have been reported (Blaak et al., 2014; Ye et al., 2017). This highlights the potential role that the agricultural environment may have as a reservoir of MDR opportunistic pathogens in fresh produce production. However, the importance of not only assessing the agricultural environment as a possible source of

antimicrobial contamination in fresh produce, but also the processing and distribution steps were discussed in a recent review (Hölzel et al., 2018). Accordingly, all ESBL-producing isolates from spinach (n=18) in the second production scenario of this study were isolated from produce during processing and retail (distribution), of which 94.4 % showed a MDR phenotype. Interestingly, from the supplier farm where no isolates were found in the agricultural environment, resistance against a maximum of one additional non-β-lactam antibiotic class was seen in the MDR ESBL-producing environmental strains, contrasting the majority of resistance profiles from the other supply chains in this study.

Molecular characterisation of the MDR ESBL/AmpC-producing Enterobacteriaceae isolates from both spinach production scenarios revealed the dominance of *bla*$_{\text{CTX-M}}$, followed by *bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM}}$. Worldwide SHV, TEM and CTX-M β-lactamases are the major ESBLs detected in clinical and agricultural settings, including fresh produce (Njage and Buys, 2014, Zhang et al., 2015; Ye et al., 2017). The most common variants reported in literature to date include *bla*$_{\text{CTX-M-14}}$ (CTX-M Group 9) and *bla*$_{\text{CTX-M-15}}$ (CTX-M Group 1). In our study, CTX-M group 9 (*bla*$_{\text{CTX-M-14}}$) was found in *E. coli* isolates from river irrigation water as well as the holding dam borehole water. This corresponds to *E. coli* isolates from river water reported by Njage and Buys (2014). Interestingly, for the CTX-M Group 1 ESBLs detected in our study, variants found in the first processing scenario included *bla*$_{\text{CTX-M-1}}$ and *bla*$_{\text{CTX-M-15}}$ from *E. coli*, *K. pneumoniae* and *S. fonticola* isolated from river, irrigation pivot point water, harvested- and retailed spinach samples, whilst in the second processing scenario, CTX-M Group 1 variants included *bla*$_{\text{CTX-M-3}}$, *bla*$_{\text{CTX-M-206}}$ and *bla*$_{\text{CTX-M-12}}$ from *S. fonticola* and *E. asburiae* isolated from spinach samples during processing and at retail. Previous studies have reported *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-15}}$ as the most broadly dispersed in clinical isolates, whilst in environmental isolates, CTX-M Group 1 variants (*bla*$_{\text{CTX-M-1}}$ and *bla*$_{\text{CTX-M-3}}$ among other), have been reported (Cantón et al., 2012; Borgogna et al., 2016).

Additionally, CTX-M Group 1 variants (*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CTX-M-3}}$ and *bla*$_{\text{CTX-M-12}}$) found in the different Enterobacteriaceae isolates from vegetables corresponded to other studies (Ye et al., 2017,

Richter et al., 2019). Apart from the ESBL genes, pAmpC resistance genes were also detected in six *S. fonticola* isolates from the second production scenario, but only included the CIT type (identified as *bla*$_{CMY}$ variants). This is in contrast to our previous findings in produce at the point of sale where the EBC type was predominantly detected from different Enterobacteriaceae species (Richter et al., 2019), but corresponds to a study by Njage and Buys (2014), who predominantly detected the CIT type pAmpC β-lactamases in *E. coli* isolated from lettuce and irrigation water samples in the North West Province, SA.

A high percentage of the ESBL/AmpC-producing isolates in the current study further harboured integrons, which is consistent with previous reports (Ben Said et al., 2015; Ye et al., 2017a). Class 1 integrons were detected in 47.96 % of the MDR ESBL/AmpC-producing isolates from both scenarios, corresponding to results reported (Ma et al., 2017; Ye et al., 2017a). Similar to results reported by Freitag et al. (2018), no class 2 integrons were detected in the current study. This contrasts to previous studies where class 2 integrons were predominantly detected, followed by class 1 integrons from raw salad vegetables retailed in Canada (Bezanson et al., 2008). In this study it was interesting that class 3 integrons were the most prevalent, detected in 72.92 % (35/48) ESBL/AmpC-producing isolates. This contrasts previous studies where only class 1 integrons were detected from water and retail food samples (Ye et al., 2017a). Co-existence of *IntI1* and *IntI3* was determined in 41.67 % (20/48) of the environmental and potential pathogenic isolates from water and spinach samples in production scenario 1 and *S. fonticola* isolates from processed and retail spinach in production scenario 2, which is a higher occurrence than the 2.9 % reported by Kargar et al. (2014) in *E. coli* isolates from a clinical setting. To the best of our knowledge, the only report of class 3 integron detection from vegetables was in a *K. pneumoniae* isolate (Jones-Dias et al., 2016). Identification of class 3 integrons have further been associated with less than ten Enterobacteriaceae genera in isolates of environmental (*Enterobacter* and *Delftia*) and clinical (*Serratia*, *Klebsiella*, and *Escherichia*) origin (Barraud et al., 2013; Jones-Dias et al., 2016;

Rajkumari et al., 2018). In our study, class 3 integrons were predominantly detected in the environmental *S. fonticola* isolates throughout each of the supply chains. Future studies will include characterisation of these integrons for determination of the gene cassettes encoding specific resistance genes present and the potential role that this class of integrons and ESBL/AmpC-producing environmental Enterobacteriaceae have in the spread of resistance genes in the agroecosystem.

6.5 Conclusion

This is the first study to show the presence of ESBL/AmpC-producing Enterobacteriaceae in the agricultural environment, throughout processing, and the retailer spinach samples. Where river water was used for irrigation, higher contamination levels were seen in the fresh produce supply chains, including an increase in ESBL/AmpC-producing Enterobacteriaceae genera isolated, as well as the phenotypic multidrug resistance profiles. This highlights the importance of the microbiological quality of irrigation water used for fresh produce to be eaten raw. Furthermore, in both spinach production scenarios, the abundance and diversity of ESBL/AmpC-producing Enterobacteriaceae on retailer spinach samples increased. This study showed that Enterobacteriaceae with expanded spectrum antimicrobial resistance are prevalent in selected fresh produce supply chains and moreover, that the resistance genes persist, with ESBL/AmpC-producing MDR organisms remaining present on fresh produce throughout processing in different production systems. The prevalence of MDR ESBL/AmpC-producing Enterobacteriaceae harbouring class 1 and class 3 integrons throughout complete spinach production systems highlights the importance of further surveillance of antimicrobial resistance in different environmental settings. In addition, this study adds to the global knowledge base regarding the prevalence and characteristics of ESBL/AmpC-producing Enterobacteriaceae in fresh vegetables and the agricultural environment required for future risk analysis. The use of whole genome sequencing for surveillance of antimicrobial resistance within the one health framework is increasingly

implemented. Future work should therefore include whole genome sequence analysis for in-depth molecular characterisation of multidrug resistant potential pathogenic isolates within the agricultural environment. This will be addressed in Chapter 7.

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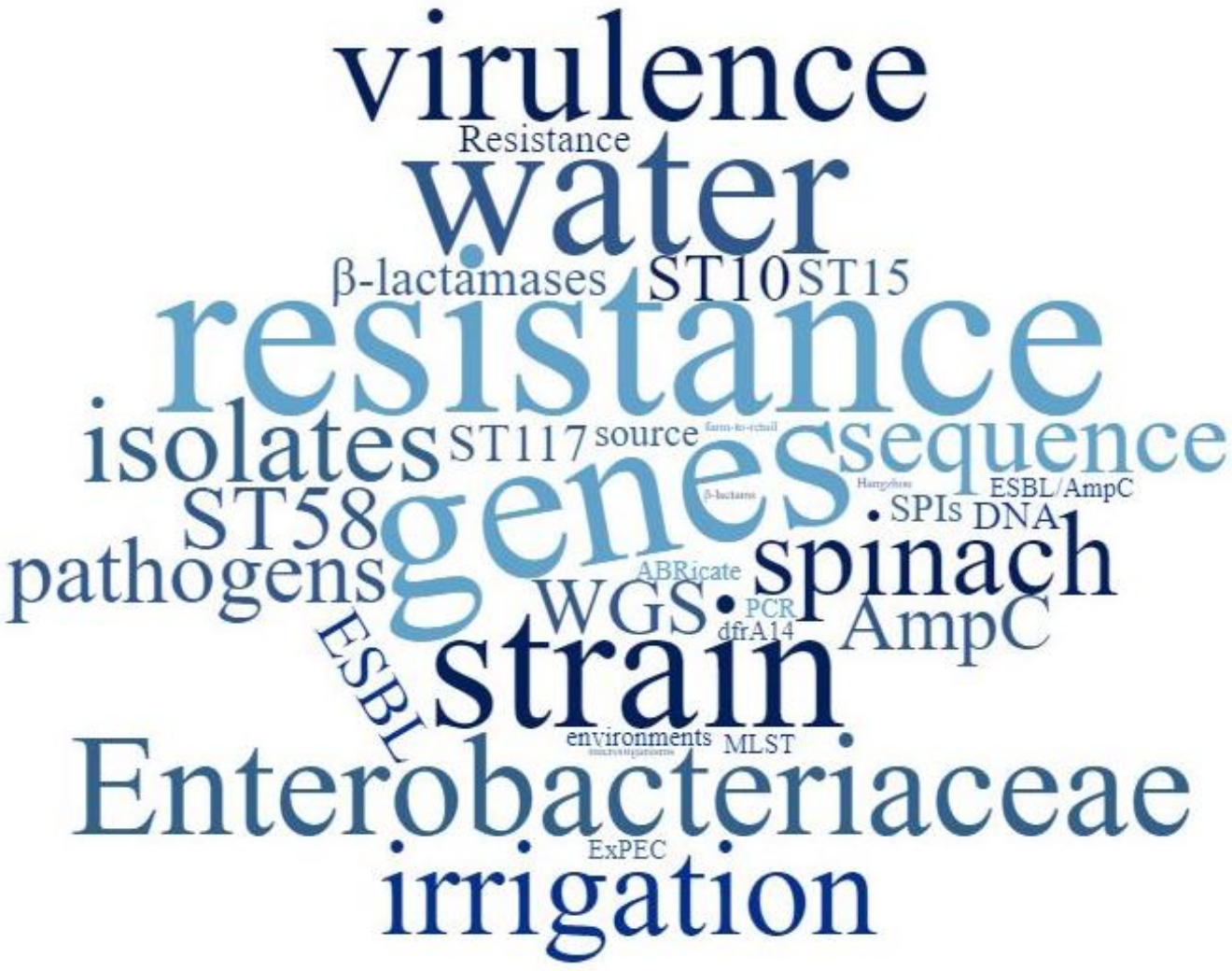
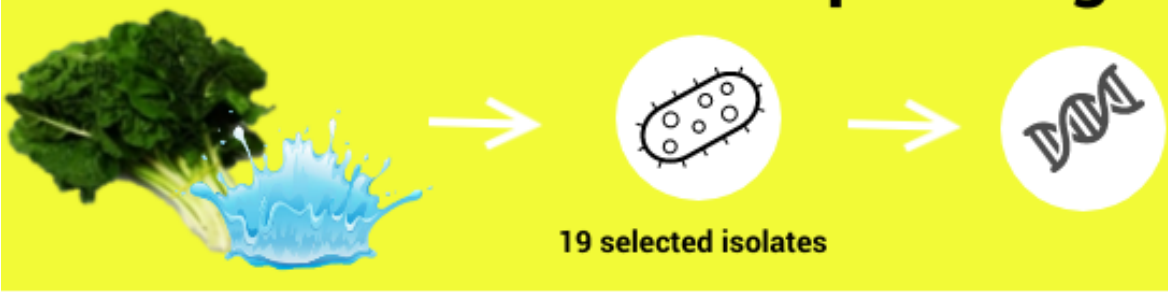
Chapter 7

"You do not know what you will find, you may set out to find one thing and end up by discovering something entirely different." -*Alexander Fleming*



Whole Genome Sequencing



- Characterisation of multidrug resistant ESBL/AmpC-producing
  Escherichia coli (n=3)
  Klebsiella pneumoniae (n=5)
  Serratia fonticola (n=10)
  Salmonella enterica (n=1)

- $bla_{CTX\text{-}M\text{-}15}$ the dominant ESBL encoding gene
- bla_{ACT} the dominant AmpC encoding gene
- Integron In191 present in six isolates

A greater number of resistance genes across more antibiotic classes in all the *K. pneumoniae* strains, compared to the other genera tested.

ESBL-producing *K. pneumoniae* ST15, an emerging high-risk clone causing nosocomical outbreaks worldwide, was isolated from irrigation water.

K. pneumoniae ST985 present in spinach at harvest and retail samples after processing, suggesting successful persistence of these multidrug resistant strains.

Isolates represent potential pathogenic genera listed by the WHO as a priority for surveillance of antimicrobial resistance screening and had confirmed similarity to human pathogens

First WGS analysis study of MDR ESBL/AmpC-producing *E. coli, K. pneumoniae, S. fonticola* and *S. enterica* isolated from spinach production systems within Gauteng Province South Africa.

Richter, L., du Plessis, E. M., Duvenage, S., Allam, M., Ismail, A., and Korsten, L. (2021). Whole Genome Sequencing of Extended-Spectrum- and AmpC- β-Lactamase-Positive Enterobacterales Isolated From Spinach Production in Gauteng Province, South Africa. 12:734649. doi: 10.3389/fmicb.2021.734649.

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Whole genome sequencing of extended-spectrum- and ampc- β-lactamase producing enterobacteriaceae isolated from spinach production in Gauteng Province, South Africa[7]

Abstract

The increasing occurrence of multidrug-resistant (MDR) extended-spectrum β-lactamase- (ESBL) and/or AmpC β-lactamase- (AmpC) producing Enterobacteriaceae in irrigation water and associated irrigated fresh produce, represent risks related to environment, food safety and public health. In South Africa, information about the presence of ESBL/AmpC-producing Enterobacteriaceae from non-clinical sources is limited, particularly in the water-plant-food interface. This study aimed to characterise 19 selected MDR ESBL/AmpC-producing *Escherichia coli* (n=3), *Klebsiella pneumoniae* (n=5), *Serratia fonticola* (n=10) and *Salmonella enterica* (n=1) isolates from spinach- and associated irrigation water samples from two commercial spinach production systems within South Africa, using whole genome sequencing (Illumina MiSeq). Antibiotic resistance genes potentially encoding resistance to eight different classes were present following analysis with ABRicate, with $bla_{\text{CTX-M-15}}$ the dominant ESBL encoding gene and bla_{ACT} the dominant AmpC encoding gene detected. A greater number of resistance genes across more antibiotic classes were seen in all the *K. pneumoniae* strains, compared to the other genera tested. From one farm, $bla_{\text{CTX-M-15}}$ positive *K. pneumoniae* strains of the same sequence type (ST 985) were present in spinach at harvest and retail samples after processing, suggesting successful persistence of these MDR strains. In addition, ESBL-producing *K. pneumoniae* ST15, an emerging high-risk clone causing nosocomical outbreaks worldwide, was isolated from irrigation water. Known resistance plasmid replicon types of Enterobacteriaceae including IncFIB, IncFIA, IncFII, IncB, and IncHI1B were observed in all strains following analysis with PlasmidFinder. However, $bla_{\text{CTX-M-15}}$ was the only β-lactamase resistance gene associated with plasmids (IncFII and

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IncFIB) in *K. pneumoniae* (n=4) strains. In one *E. coli* and five *K. pneumoniae* strains, integron In191 were observed. Relevant similarity to human pathogens were predicted with PathogenFinder for all 19 strains, with a confidence of 0.635- 0.721 in *S. fonticola*, 0.852 – 0.931 in *E. coli*, 0.796 – 0.899 in *K. pneumoniae* and 0.939 in the *S. enterica* strain. The presence of MDR ESBL/AmpC-producing *E. coli*, *K. pneumoniae*, *S. fonticola* and *S. enterica* with confirmed similarities to human pathogens that reflect the agricultural production environment link in the emergence and spread of antibiotic resistance genes.

7.1 Introduction

The discovery of antibiotics in the 1940's led to a new age in medical care. However, the global increase in antimicrobial resistance (AMR) is reducing the effectiveness of clinically important antibiotics (Lobanovska and Pilla, 2017; Dandachi et al., 2019). An example of shifting resistance profiles in bacteria are within the β-lactam class of antibiotics, including penicillins and third generation cephalosporins, which are the most widely used in human and veterinary medicine and widely expressed AMR are being reported (Finton et al., 2020). Persistent exposure to these antibiotics have resulted in bacteria becoming resistant by evolving extended-spectrum β-lactamases (ESBLs), which hydrolyze the β-lactam ring within the antibiotic. Thus rendering it inactive (Bush and Jacoby, 2010). Consequently, production of ESBLs are regarded as one of the most clinically significant resistance mechanisms (Bush and Jacoby, 2010), with ESBL-producing Enterobacteriaceae (*Escherichia coli*, *Klebsiella pneumoniae* and *Serratia* spp., among others) listed as priority pathogens for research and development in the new frontier of antibiotics [World Health Organisation (WHO), 2017].

Classified into several groups according to their amino acid sequence homology, the CTX-M, TEM and SHV ESBL variants are the most common β-lactamases identified in Enterobacteriaceae (van Duin and Doi, 2017). In addition, AmpC β-lactamases are chromosomally encoded by several

Enterobacteriaceae species and play a key role in resistance development (van Duin and Doi, 2017). Plasmid encoded AmpC genes have been known since 1989 (Jacoby, 2009) and are now regularly reported in clinical and environmental strains (Khari et al., 2016; Colosi et al., 2020; Tekele et al., 2020). Both chromosomally encoded and plasmid-mediated AmpC β-lactamases confer resistance to a broad spectrum of β-lactams such as penicillins, oxyimino-cephalosporins (including cefotaxime and ceftazidime), cephamycins and aztreonam at variable levels (Jacoby, 2009; Palzkill, 2018).

The increase in antimicrobial resistant strains and effective resistance mechanisms among Enterobacteriaceae has led to numerous global reports of ESBLs, AmpC-, and more recently carbapenemase-producing Enterobacteriaceae not only in clinical settings, but also in the agricultural environment (Ye et al., 2017b; Al-Kharousi et al., 2019; Dandachi et al., 2019; Hassen et al., 2020; Richter et al., 2020). Although members of the Enterobacteriaceae family occur naturally in human and animals' gastrointestinal tracts as well as in the environment (water, soil and plants) (Blaak et al., 2014c; Ye et al., 2017b), occurrence of multidrug resistant (MDR) strains in the different habitats are concerning. Inadequately treated or untreated effluents from industries, households and zootechnical farms are reported as one of the main contamination causes of South African surface- and ground water resources (Verlicchi and Grillini, 2020). It is also well documented that the three principal antibiotic contamination channels in the environment are animal-, human- and manufacturing waste (O'neill, 2016). Consequently, contamination of soil, irrigation- and drinking water as well as crops can occur, adding additional exposure routes to humans (Finton et al., 2020).

Previous surveillance studies have shown prevalence of MDR ESBL/AmpC-producing Enterobacteriaceae in fresh vegetables sold in South Africa (Richter et al., 2019) and in other countries i.e the Netherlands, Switzerland and Germany (Reuland et al., 2014a; Zurfluh et al., 2015; Reid et al., 2020). Occurrence of ESBL-producing Enterobacteriaceae have also been reported in corresponding irrigation water sources and cultivated crops (Blaak et al., 2014c; Njage and Buys, 2014; Ye et al.,

2017b). Furthermore, Richter et al. (2020) reported occurrence of ESBL/AmpC-producing Enterobacteriaceae in different spinach supply chains from irrigation water and produce at harvest, throughout processing and at retail in the Gauteng Province of South Africa.

The high discriminatory power of whole genome sequencing (WGS) has led to an increase in use of this method for detecting points of contamination, source tracking, pathogen surveillance and outbreak investigations [Oniciuc et al., 2018; Centre for Disease Control and Prevention (CDC), 2019]. Whole genome sequencing provides information regarding multiple antimicrobial resistance genes, genomic mutations, mobile genetic elements and association with resistance genes, as well as higher-resolved microbial typing (Oniciuc et al., 2018a; CDC, 2019; Kim et al., 2020). Consequently, the WGS results can aid in elucidating the genetic relationship among isolates from different environments and along the food chain (Adator et al., 2020). Surveillance of antimicrobial resistant strains through WGS is increasingly being used due to increasing accessibility and affordability (Adator et al., 2020). In South Africa, WGS has been used for characterisation of clinical ESBL-producing *K. pneumoniae* strains among others (Founou et al., 2019), as well as typing of *Listeria monocytogenes* from environmental and clinical settings during the 2017 listeriosis outbreak (Thomas et al., 2020). However, the use of WGS for surveillance of antimicrobial resistant potential pathogenic Enterobacteriaceae in retailed fresh produce and the production environment, have not been reported locally.

The World Health Organisation (WHO) developed Global Antimicrobial Resistance Surveillance System (GLASS) in 2015 supporting research and surveillance as well as a global data sharing through a standardized analysis approach (WHO, 2020). Initially, the GLASS focus was mainly on surveillance of human priority pathogens, but has since shifted to include AMR in foodborne pathogens (WHO, 2020). Moreover, the one health framework for understanding AMR in pathogenic Gram- negative bacteria, is increasingly attracting attention (Collignon and McEwen, 2019). In SA information regarding AMR in fresh produce production systems and specifically focusing on the

Enterobacteriaceae is lacking. The aim of this study was thus to use whole genome sequencing for analysis of AMR genes, associated mobile genetic elements, virulence factors, serotypes, multi-locus sequence types and pathogenicity of selected, partially characterised, ESBL/AmpC-producing environmental Enterobacteriaceae from commercial spinach production systems (Richter et al., 2020). These isolates included four different species (*E. coli*, *K. pneumoniae*, *Serratia fonticola* and *Salmonella enterica*) listed by the WHO as a particular threat of Gram-negative bacteria that are resistant to multiple antibiotics (WHO, 2017), while isolates harbouring integrons as described in Richter et al. (2020) were preferentially selected. The results of this study will contribute towards the global knowledge base and understanding of how genetic processes within the water-plant-food interface might impact human health and disease.

7.2 Materials and Methods

7.2.1 Sample collection, isolation and DNA extraction of extended-spectrum β-lactamase and AmpC-producing Enterobacteriaceae

Irrigation water and fresh produce samples from spinach production systems were collected and ESBL-producing Enterobacteriaceae were isolated as described in Chapter 6 (Richter et al. 2020). A selection of 19 isolates were further characterized (Table 7.1). The genomic DNA of each isolate was extracted with the DNeasy PowerSoil kit (Qiagen, South Africa) according to the manufacturer's instructions. Following gDNA extraction, the concentrations were determined using the Qubit dsDNA Broad Range Assay and a Qubit 2.0 fluorometer (Life Technologies, Johannesburg) and quantification was determined on a Nanodrop 2000 (ThermoScientific, Johannesburg).

Table 7.1: Isolates selected for whole genome sequence analysis from the agricultural environment in spinach supply chains, Gauteng Province, South Africa

Strain	Organism identity	Source water (W) or spinach (S)	Isolation point from spinach production systems
UPMP2117	*Escherichia coli*	W	Water reservoir
UPMP2120	*Escherichia coli*	S	Unwashed spinach bunches at retailer
UPMP2130	*Escherichia coli*	W	Holding dam water (source water)
UPMP2112	*Klebsiella pneumoniae*	W	Irrigation pivot point water
UPMP2114	*Klebsiella pneumoniae*	S	Spinach at harvest
UPMP2118	*Klebsiella pneumoniae*	W	Irrigation pivot point water
UPMP2121	*Klebsiella pneumoniae*	S	Unwashed spinach bunches at retailer
UPMP2122	*Klebsiella pneumoniae*	S	Spinach at retailer
UPMP2115	*Salmonella spp.*	W	River water
UPMP2116	*Serratia fonticola*	W	River water
UPMP2119	*Serratia fonticola*	W	Irrigation pivot point water
UPMP2123	*Serratia fonticola*	S	Unwashed spinach punnet at retailer
UPMP2124	*Serratia fonticola*	S	Spinach at receival
UPMP2125	*Serratia fonticola*	S	Spinach after pack
UPMP2126	*Serratia fonticola*	S	Spinach at receival
UPMP2127	*Serratia fonticola*	S	Unwashed spinach at retailer
UPMP2128	*Serratia fonticola*	S	Unwashed spinach at retailer
UPMP2129	*Serratia fonticola*	S	Spinach at receival
UPMP2131	*Serratia fonticola*	S	Unwashed spinach at retailer

7.2.2 DNA sequencing and whole genome analysis

Sequencing was performed on an Illumina MiSeq instrument with 100X coverage by the National Institute for Communicable Diseases Sequencing Core Facility, South Africa, following preparation of multiplexed paired-end libraries (2x300bp) with the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA). The resultant reads were quality trimmed using CLC version 20 (https://digitalinsights.qiagen.com) and *de novo* assembled with all assembly metrics shown in Appendix F, Table F1. The contiguous sequences were then submitted to the National Centre for Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline (https://pubmed.ncbi.nlm.nih.gov/27342282/). Antimicrobial resistance gene presence was corroborated using ABRicate (https://github.com/tseemann/abricate) that included the Comprehensive Antibiotic Resistance Database (CARD), ARG-ANNOT, ResFinder, NCBI AMRFinder Plus, and MEGARes databases (Zankari et al., 2012; Gupta et al., 2014; Jia et al., 2017; Feldgarden et al., 2019; Doster et al., 2020).

Plasmid replicon types were determined with PlasmidFinder (version 2.1) (https://cge.cbs.dtu.dk/services/) (Carattoli et al., 2014). Using the Centre for Genomic Epidemiology (CGE) platform (https://cge.cbs.dtu.dk/services/), mobile genetic elements for all four species, sequence types of *E. coli*, *K. pneumoniae* and *S. enterica* as well as the *E. coli* serotypes based on lipopolysaccharide (O-antigen) and capsular flagella (protein) (H-antigen) and virulence genes of *E. coli* were determined with MGEFinder, Multilocus Sequence Typing (MLST) (version 2.2), SeroTypeFinder (version 2.0) and VirulenceFinder (version 2.0), respectively (Larsen et al., 2012; Joensen et al., 2014, 2015; Johansson et al., 2021). The following parameters were used in the Serotype Finder Web-based tool: 85% threshold for %ID and 60% minimum length (the number of nucleotides in a sequence of interest that must overlap a serotype gene to count as a hit for that gene) (Joensen et al., 2015).

The *in silico* serotyping based on the capsule polysaccharide (K-antigen) of *K. pneumoniae* strains were conducted using Kaptive Web (Wick et al., 2018), whilst the presence of virulence genes for *K. pneumoniae* were identified by using the Institut Pasteur's *Klebsiella* database (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html). Additionally, paired reads of the whole genome sequencing raw data files for the *S. enterica* strain was uploaded to the online SeroSeq tool version 1.0 which predicted the *Salmonella* serotype of the requested isolate (Zhang et al., 2015; Thompson et al., 2018). The *Salmonella* Pathogenicity Islands (SPI) were identified with SPIFinder 2.0 (Roer et al., 2016). Next, the existence of virulence factors in each SPI were analysed by performing BLAST analysis on the predicted SPIs against the virulence factor database (VFDB) (Chen et al., 2016; Ashari et al., 2019). The virulence factors of *S. fonticola* and were determined using the VFDB with ABRicate (Chen et al., 2016). All sequences were submitted to the INTEGRALL database (http://integrall.bio.ua.pt) for annotation and integron number assignment. Using PathogenFinder (version 1.1) on the CGE platform (https://cge.cbs.dtu.dk/services/PathogenFinder/), the strains' pathogenicity towards humans were predicted (Cosentino et al., 2013).

7.2.3 Data availability

The nucleotide sequences of the 19 Enterobacteriaceae strains described in this paper were deposited in the National Center for Biotechnology Information GenBank database in the BioProject number: PRJNA642017, accession numbers NZ_JACAAL010000000, NZ_JACBIV000000000-NZ_JACBJE000000000 and NZ_JACNYM000000000-NZ_JACNYT000000000.

7.3 Results

7.3.1 Detection of antimicrobial resistance genes

The selected 19 ESBL/AmpC producing Enterobacteriaceae isolates all harboured at least one β-lactamase encoding gene in addition to the ESBL/AmpC genetic determinants, accompanied by

resistance genes from different antibiotic classes including fluoroquinolone, sulfonomide, fosfomycin, aminoglycoside, trimethroprim, phenicol and/or tetracycline (Figure 7.1). The β-lactamase resistance genes included chromosomally encoded AmpC in the *S. enterica* strain as well as all three *E. coli* strains. Plasmid-mediated AmpC genes (*bla*$_{\text{CMY-113}}$ and *bla*$_{\text{CMY-101}}$) were present in two *E. coli* strains from irrigation water and *bla*$_{\text{ACT-13}}$, *bla*$_{\text{ACT-38}}$, *bla*$_{\text{ACT-6}}$ and/or *bla*$_{\text{ACT-58}}$ were present in ten *S. fonticola* strains from irrigation water (n=2) and spinach (n=8) samples (Figure 1). Additionally, *bla*$_{\text{FONA-5}}$ (n = 8) from irrigation water and spinach and *bla*$_{\text{FONA-6}}$ (n = 2) from spinach were present in *S. fonticola* strains. The ESBL genes included *bla*$_{\text{SFO-1}}$ in all ten *S. fonticola* strains, *bla*$_{\text{CTX-M-15}}$ in five *K. pneumoniae* strains from irrigation water and spinach, and one *E. coli* strain from spinach. It also included *bla*$_{\text{CTX-M-14}}$ in an *E. coli* strain from irrigation water, whilst *bla*$_{\text{SHV-187}}$ (n = 3), *bla*$_{\text{SHV-106}}$ (n = 1) and *bla*$_{\text{SHV-178}}$ (n = 1) were present in *K. pneumoniae* strains (Figure 7.1).

Interestingly, a greater number of resistance genes across more classes were seen in all the *K. pneumoniae* strains (n=5), compared to the other genera tested. All five *K. pneumoniae* strains had chloramphenicol (*catB3*), aminoglycosides [*aac(6')-Ib-cr*, *aph(6)-Id* and *aph(3'')-Ib*], fosfomycin (*fosA6*) and sulfonomide (*sul2*) resistance genes present (Figure 7.1). Other resistance genes included fluoroquinolone *oqxA* (n = 4), *oqxB* (n = 4), and *qnrB1* (n = 4) in *K. pneumoniae* from spinach and water, *qnrS1* (n = 1) in *E. coli* from spinach and *qnrB6* (n = 3), *qnrB37* (n = 5), *qnrE1* (n = 10) in *S. fonticola* from spinach and water, whilst *mdtk* (n = 4), and *mdtH* (n = 3) were present in *S. fonticola* from water only. The *qnrB17* resistance gene were present in *K. pneumoniae* (n=4) and *S. fonticola* (n=2) strains from spinach and water (Figure 7.1). The *S. enterica* strain isolated from irrigation water also harboured *aac(6')-Iaa* and *aac(6')-Iy* aminoglycoside resistance genes (Figure 7.1) and a *S. fonticola* strain from irrigation water harboured an aminoglycoside [*aph(3'')-Ib*] and sulfonomide (*sul2*) resistance gene (Figure 7.1).

7.3.2 Detection of mobile genetic elements and association to antimicrobial resistance genes

Known resistance plasmid replicon types of Enterobacteriaceae including IncFIB, IncFIA, IncFII, IncB, and IncHI1B were observed in all strains following analysis with PlasmidFinder (data not shown). The β-lactamase gene, *bla*$_{\text{CTX-M-15}}$, was the only resistance gene associated with plasmids (IncFII_pKP91 and/or IncFIB(K)_1_Kpn3) in four *K. pneumoniae* strains upon further analysis (Table 7.2). The IS*6* family elements (IS6100) have been reported to play a pivotal role in the dissemination of resistance determinants in Gram-negative bacteria (Partridge et al., 2018), and were observed in relation to the *dfrA14b* resistance gene in all five *K. pneumoniae* strains (Table 7.2). The *bla*$_{\text{CTX-M-14}}$ and *sul2* resistance genes were related to the IScEP1 element within the IS1380 family in one *E. coli* and three *K. pneumoniae* strains, respectively, whilst one *S. fonticola* strain carried a *sul2* gene that was related to IS110 (Table 7.2). One *E. coli* strain carried *bla*$_{\text{CTX-M-15}}$ that was related to ISKra4. Other insertion sequences detected belonged predominantly to the IS3 and IS110 families (data not shown), with one *K. pneumoniae* strain carrying the *bla*$_{\text{SHV-80}}$ broad spectrum β-lactamase that was related to IS3 (Table 7.2). In all *K. pneumoniae* strains (n=5) where the *qnrB1* resistance gene was present, association to Tn5403 were seen (Table 7.1). In one *E. coli* and five *K. pneumoniae* strains, integron In191 was observed, with *dfrA14* in the cassette array (Table 7.2).

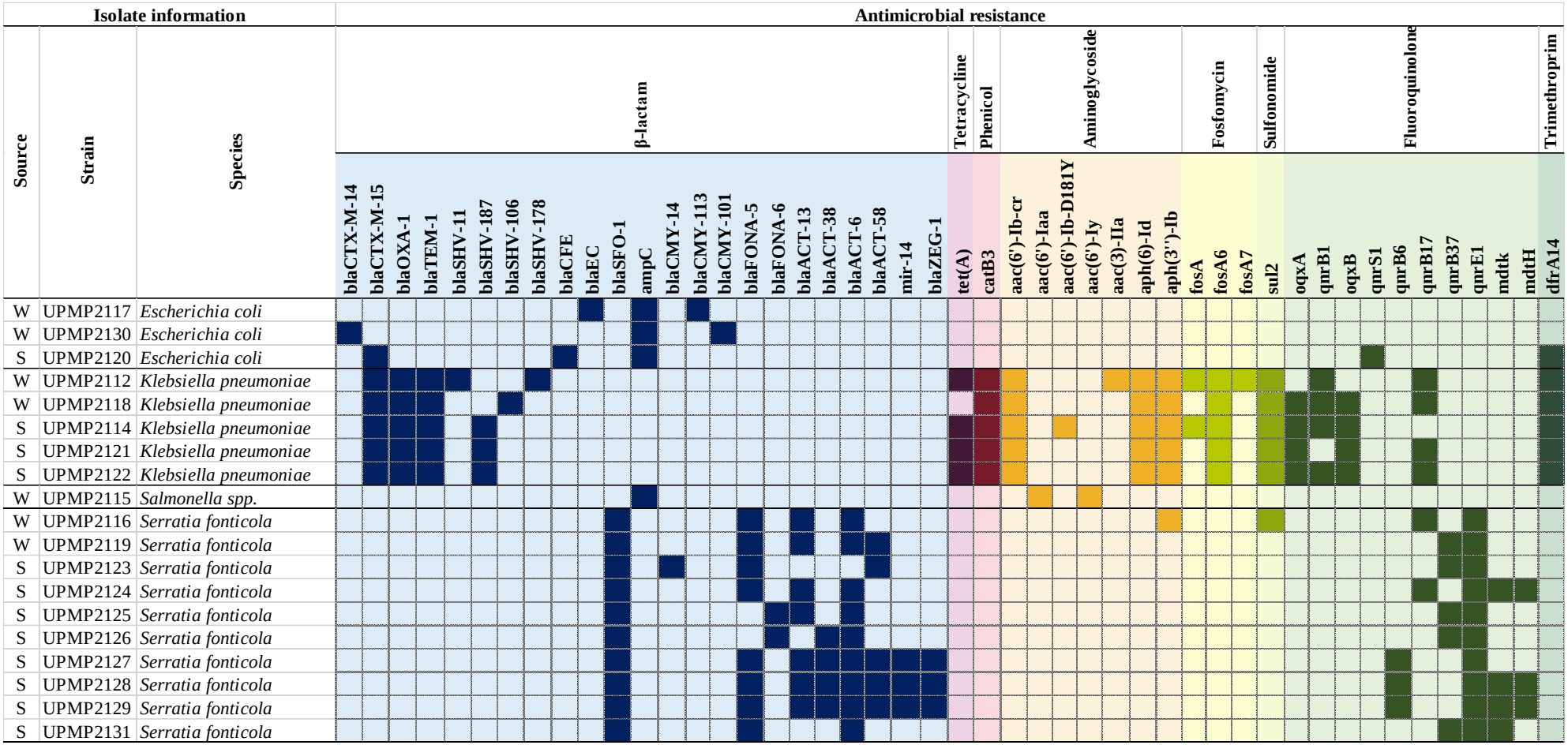

Figure 7.1: Antimicrobial resistance genes present in Enterobacteriaceae isolated from water and spinach from farm to retail. Abbreviations: Water (W) and Spinach (S)

7.3.3 *In silico* analysis of serotypes, multi-locus sequence types and virulence factors

The *in silico* MLST analysis, predicted serotypes and pathogenicity probability of all 19 strains, are shown in Table 7.3. Three different sequence types (ST58, ST117, and ST10) and three different serotypes (O75:H9, O11:H4, and O8:H17) were observed in the three *E. coli* strains. The five *K. pneumoniae* strains belonged to three different sequence types and three different serotypes (KL27, KL24, and KL39) which were observed based on the K-antigen, whilst the O-serotype included O4 and O1 (Table 7.3). The predicted antigenic profile of the *S. enterica* strain was O11:k:1,2. Furthermore, the *S. enterica* strain contained 11 Salmonella SPI, namely SPI-1, SPI-2, SPI-3, SPI-4, SPI-5, SPI-9, SPI-13, SPI-14, one unnamed, as well as the centisome 63 (C63PI) and 54 (CS54) pathogenicity islands, each harbouring between 20 and 60 virulence factors (Appendix F Table F2). A total of 42 virulence genes were identified in the *E. coli* and *K. pneumoniae* strains (Appendix F Table F3 and F4). Of these, 20 were detected in *E. coli* strains only and 20 in *K. pneumoniae* strains only, whilst *fyuA* and *irp2* virulence factors were detected in two *E. coli* strains from irrigation water as well as three *K. pneumoniae* strains from spinach samples. All three *E. coli* strains carried the *terC* virulence gene (Appendix F Table F3) and in all five *K. pneumoniae* strains, the *mrkA*, *mrkB*, *mrkC*, *mrkD*, *mrkE*, *mrkH* and *mrkI* virulence factors were present (Appendix F Table F3). No shiga-toxin producing genes were present in the *E. coli* strains. A total of 89 virulence factors were identified in the *S. fonticola* strains (Appendix F Table F4). This included 25, 18, 16, and 6 of the virulence factors present in 100% (n=10), 90%, 80%, and 70% of the selected *S. fonticola* strains, respectively, whilst the remaining 24 virulence factors were present in varying numbers in one to six of the strains (Appendix F Table F4). The *iroN* salmochelin siderophore receptor which plays a role in disease establishment was present in three *S. fonticola* strains (two from unwashed baby spinach samples at the retailer and one from the irrigation pivot point water), one *E. coli* strain from the ground water, as well as in the SPI-13 in the *S. enterica* strain from river irrigation water. Relevant similarity to human pathogens were predicted for all 19 strains with a confidence of 0.635-

0.721 in the *S. fonticola* strains (n=10), 0.852 – 0.931 in the *E. coli* strains (n=3), 0.796 – 0.899 in the *K. pneumoniae* strains (n=5) and 0.939 in the *S. enterica* strain. (Table 7.3).

Table 7.2: Extended spectrum β-lactamase and AmpC-producing Enterobacteriaceae with resistance genes related to mobile genetic elements

Isolate information			Resistance genes associated with mobile genetic elements					
			Genes		Mobile genetic elements			
Source	**Strain**	**Species**	**β-lactamase**	**Other**	**Plasmids**	**Insertion sequences**	**Transposons**	**Integron**
W	UPMP2130	*Escherichia coli*	CTX-M-14			IS1380		
S	UPMP2120	*Escherichia coli*	CTX-M-15	dfrA14b		ISKra4		In191
W	UPMP2112	*Klebsiella pneumoniae*	SHV-80 CTX-M-15	sul2 qnrB1 dfrA14b	IncFIB(K)_1_Kpn3	IS3 IS1380 IS6	Tn5403	In191
W	UPMP2118	*Klebsiella pneumoniae*	TEM-1B	dfrA14b qnrB1		IS1380 IS6	Tn5403	In191
S	UPMP2114	*Klebsiella pneumoniae*	CTX-M-15	sul2 qnrB1 dfrA14b	IncFII_pKP91 IncFIB(K)_1_Kpn3	IS1380 IS6	Tn5403	In191
S	UPMP2121	*Klebsiella pneumoniae*	CTX-M-15 TEM-1B	qnrB1 dfrA14b	IncFII_pKP91	IS1380 IS6	Tn5403	In191
S	UPMP2122	*Klebsiella pneumoniae*	CTX-M-15	sul 2 qnrB1 dfrA14b	IncFII_pKP91 IncFIB(K)_1_Kpn3	IS1380 IS6	Tn5403	In191
W	UPMP2116	*Serratia fonticola*		sul2		IS110		

Abbreviations: Water (W) and Spinach (S)

Table 7.3: *In silico* multilocus sequence typing analysis, predicted serotypes and pathogenicity probability of Enterobacteriaceae isolated from irrigation water and spinach throughout production from farm to retail

Accession	Strain	Source	Species	Sequence type	Serotype	Pathogenicity probability
NZ_JACNYS000000000	UPMP2120	S	*Escherichia coli*	ST58	O75:H9	0.888
NZ_JACNYT000000000	UPMP2117	W	*Escherichia coli*	ST117	O11:H4	0.931
NZ_JACNYN000000000	UPMP2130	W	*Escherichia coli*	ST10	O8:H17	0.852
NZ_JACAAL010000000	UPMP2112	W	*Klebsiella pneumoniae*	ST3559	KL27:O4	0.899
NZ_JACBJB000000000	UPMP 2118	W	*Klebsiella pneumoniae*	ST15	KL24:O1v1	0.889
NZ_JACBJE000000000	UPMP2114	S	*Klebsiella pneumoniae*	ST985	KL39:O1v2	0.885
NZ_JACBIZ000000000	UPMP2121	S	*Klebsiella pneumoniae*	ST985	KL39:O1v2	0.796
NZ_JACBIY000000000	UPMP2122	S	*Klebsiella pneumoniae*	ST985	KL39O1v1	0.885
NZ_JACBJD000000000	UPMP2115	W	*Salmonella enterica*	ST4924	Pretoria	0.939
NZ_JACBJC000000000	UPMP2116	W	*Serratia fonticola*	N.D	N.D	0.721
NZ_JACBJA000000000	UPMP2119	W	*Serratia fonticola*	N.D	N.D	0.699
NZ_JACBIX000000000	UPMP2123	S	*Serratia fonticola*	N.D	N.D	0.692
NZ_JACNYR000000000	UPMP2124	S	*Serratia fonticola*	N.D	N.D	0.635
NZ_JACNYQ000000000	UPMP2125	S	*Serratia fonticola*	N.D	N.D	0.645
NZ_JACNYP000000000	UPMP2126	S	*Serratia fonticola*	N.D	N.D	0.659
NZ_JACNYO000000000	UPMP2127	S	*Serratia fonticola*	N.D	N.D	0.659
NZ_JACBIW000000000	UPMP2128	S	*Serratia fonticola*	N.D	N.D	0.674
NZ_JACBIV000000000	UPMP2129	S	*Serratia fonticola*	N.D	N.D	0.659
NZ_JACNYM000000000	UPMP2131	S	*Serratia fonticola*	N.D	N.D	0.705

Abbreviations: Water (W) and Spinach (S), Not detected (N.D.)

7.4 Discussion

To the authors knowledge this is the first study to use WGS for in-depth molecular characterization of ESBL/AmpC-producing *E. coli, K. pneumoniae, S. enterica and S. fonticola* isolates, previously identified and partially characterized, from spinach and irrigation water samples in commercial production chains (Richter et al., 2020). Characterization included antimicrobial resistance, mobile genetic elements (e.g. insertion sequences, plasmids and integrons), serotypes and determining the pathogenicity. All these factors are crucial in defining and attributing infection sources of food-related outbreaks caused by resistant microorganisms (Oniciuc et al., 2018). Overall, results corresponded with main global findings where AMR genes and associated mobile genetic elements have been reported in Enterobacteriaceae from fresh produce and irrigation water, with the potential to pose a health risk to humans upon exposure (Jones-Dias et al., 2016b; Finton et al., 2020).

Previously, the presence of *intI3* were reported in a high percentage of isolates from the current study following conventional PCR and sequencing (Richter et al., 2020). However, in-depth WGS analysis showed that no *attI* fragment preceded the *IntI3* genes, consequently, the *IntI3* genes detected and previously reported did not form part of complete integrons, which typically include an integrase *intI* gene encoding a site-specific recombinase, a recombination site *attI* as well as a promoter (P_c) (Kaushik et al., 2018). Overall, six isolates in the current study were positive for Class 1 integrons (In191), similar to In191 positive clinical ESBL-producing Enterobacteriaceae from an academic teaching hospital in Pretoria, SA (Sekyere et al., 2020). Additionally, these MDR environmental isolates harbored various virulence factors central to pathogenicity, including genes associated with urinary tract infections and iron sequestering systems crucial for disease establishment. All isolates had relevant similarity to human pathogens and form part of the WHO 3rd generation cephalosporin resistant critical priority pathogens (WHO, 2017).

Two of the *E. coli* strains from the current study harboured plasmid-mediated AmpC $bla_{\text{CMY-2-like}}$ genes ($bla_{\text{CMY-113}}$ and $bla_{\text{CMY-101}}$), which correspond to the phenotypic profile of resistance to expanded-spectrum cephalosporins previously reported for these isolates using traditional PCR analysis (Richter et al., 2020). The $bla_{\text{CMY-2}}$ pAmpC genes are the most commonly reported in *E. coli* and other Enterobacteriaceae species and have clinical relevance, as it inactivates 3rd generation cephalosporins and mediate resistance to carbapenems (Jacoby, 2009; Bortolaia et al., 2014). Three different multi-locus sequence types, namely ST58, ST10, and ST117, were identified in the *E. coli* isolates. Isolated from the retailed unwashed spinach samples in the current study, ST58 *E. coli* have previously also been associated with human extra-intestinal infections including sepsis, and have emerged worldwide in wild and food-production animals (Reid et al., 2020). As an example, ST58 *E. coli* with serotype O75:H9 corresponded to an *E. coli* strain of bovine origin from Pakistan and also carried the IncFIB plasmid (Ali et al., 2020).

Although the strain from the current study had less AMR genes than reported in ST58 *E. coli* with serotype O75:H9 by Ali et al. (2020), the trimethoprim (*dfrA14*), fluoroquinolone (*qnrS1*) and β-

lactam (*bla*$_{CTX-M-15}$) genes corresponded. Similarly, uropathogenic ST58 *E. coli* with resistance to fluoroquinolone and trimethoprim have previously been isolated from hospital patients in Australia (McKinnon et al., 2018). The *bla*$_{CTX-M-15}$ gene identified in the ST58 *E. coli* strain from the current study was associated with the ISKra4 insertion sequence, previously identified in *K. pneumoniae* harbouring *bla*$_{CTX-M-15}$, and responsible for the movement to different parts of the genome through a replicative transposition mechanism (Razavi et al., 2020). In contrast to Hauser et al. (2013) who identified food-associated shiga-toxin producing *E. coli* ST58, no *stx* genes were present in the strains. The *E. coli* ST58 from the current study harboured the *gad* (glutamate decarboxylase) virulence gene, similar to *E. coli* ST58 strains isolated from aragula (rocket) (Reid et al., 2020). However, the presence of *lpfA* (long polar fimbriae) and *terC* (tellurium ion resistance protein) virulence factors in the strain from the current study, contrasted the virulence gene profiles reported by Reid et al. (2020). *Escherichia coli* ST10 have previously been associated with human clinical infections and has been isolated from different sources including recreational and/or wastewater samples (Falgenhauer et al., 2019). From the current study, the *E. coli* ST10 with serotype O8:H17 was isolated from borehole water used for irrigation. Although this sequence type has previously been associated with shiga-toxin-producing *E. coli* (STEC) (Gonzalez-Escalona and Kase, 2018), no *stx* genes were detected in the current study. The virulence factors present were *terC* (tellurium ion resistance protein), *astA* (EAST-1 heat-stable toxin), *fyuA* (ferric yersiniabactin uptake receptor), *irp2* (nonribosomal peptide synthetases), *iss* (increased serum survival) and *sitA* (iron transport protein). Previously, *E. coli* ST10 with similar virulence gene profiles were isolated from human blood cultures and reported as extra-intestinal pathogenic *E. coli* (ExPEC) (Maluta et al., 2017). Additionally, ESBL-producing *E. coli* ST10 of the same serotype have been isolated from wastewater and are depicted as a probable environmental reservoir of *bla*$_{CTX-M}$ genetic determinants (Tanaka et al., 2019).

In the current study, the ST58 *E. coli* strain harboured the *bla*$_{CTX-M-15}$ genetic determinant, whilst *bla*$_{CTX-M-14}$ was present in the ST10 *E. coli* strain. Globally, the CTX-M type ESBLs (especially

bla$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-15}}$) have become the dominant genotype and the most widely distributed (Cantón et al., 2012; Adamski et al., 2015). *Escherichia coli bla*$_{\text{CTX-M-14}}$ positive strains have previously been isolated from store bought produce in Germany and South Africa (Richter et al., 2019; Reid et al., 2020), food producing animals in China (Liao et al., 2015) and clinical settings in Brazil and SA (Cergole-Novella et al., 2010; Peirano et al., 2011).

The third *E. coli* sequence type (ST117) detected from irrigation source water in the current study, have previously been reported as part of a group of multi-serotype extra-intestinal pathogenic *E. coli* (ExPEC) and avian pathogenic *E. coli* (APEC) strains (Kim et al., 2017). The *E. coli* ST117 strain from the current study harboured 20 virulence factors including the ExPEC *hlyF* (Hemolysin F) virulence gene. In previous studies, *stx* genes were identified in *E. coli* strains with the same STs detected in the current study, yet the virulence gene content and serotypes differ from the strains in the current study (Gonzalez-Escalona and Kase, 2018). However, the three non-STEC *E. coli* strains (ST58, ST10, and ST117) from the current study had a 93%, 89% and 85% probability of being human pathogens, based on the pathogenic protein families.

In addition to *E. coli*, other Enterobacteriaceae isolates harbouring *bla*$_{\text{CTX-M-15}}$ have also been detected in different environments. In the current study, all five *K. pneumoniae* strains harboured the *bla*$_{\text{CTX-M-15}}$ genetic determinant. The prevalence and dissemination of *bla*$_{\text{CTX-M}}$ throughout various environments globally underlines the different contamination routes through which fresh produce may also become contaminated with these MDR organisms. For instance, Gekenidis et al. (2020) have demonstrated the long-term persistence of *E. coli* harbouring *bla*$_{\text{CTX-M-15}}$ in soil and lettuce after its introduction via irrigation water. Similarly, *bla*$_{\text{CTX-M-15}}$ positive ST985 *K. pneumoniae* strains were present in spinach at harvest on the farm as well as retail samples after processing in the current study, suggesting successful persistence of these MDR strains. In four *K. pneumoniae* strains (ST3559, n=1 and ST985, n=3), the *bla*$_{\text{CTX-M-15}}$ genes were associated with IncF replicons (IncFII$_{\text{K}}$ and IncFIB) which have previously been linked to diverse *K. pneumoniae* outbreak strains (Dolejska et al., 2012, 2013; Löhr et al., 2015). Moreover, in *K. pneumoniae*

ST3559, *bla*$_{\text{CTX-M-15}}$ was also associated with *ISEcp1* (also called *ISEc9*), a member of the widely reported IS1380 family, and can enable the independent transposition with insertion mutation and genetic relocations (Partridge, 2011). The *K. pneumoniae* strains in the current study also harboured *bla*$_{\text{SHV}}$ ESBL encoding genes (*bla*$_{\text{SHV-187}}$, *bla*$_{\text{SHV-106}}$ and *bla*$_{\text{SHV-178}}$). Previously, SHV genetic determinants were reported in *K. pneumoniae* from hospitals and receiving wastewater treatment plants in Romania (Surleac et al., 2020) as well as irrigation water and agricultural soil in SA (Iwu et al., 2020; Richter et al., 2020). Interestingly, the *K. pneumoniae* ST15 strain isolated from water in the current study harboured *bla*$_{\text{SHV-106}}$ which Liakopoulos et al. (2016) previously reported to be geographically constrained and have only been described in *K. pneumoniae* isolates from Portugal together with *bla*$_{\text{TEM-1}}$. Similarly, the *K. pneumoniae* ST15 strain from the current study also harboured *bla*$_{\text{SHV-106}}$ together with *bla*$_{\text{TEM-1}}$. *Klebsiella pneumoniae* ST15 is regarded as an emerging international high-risk clone causing nosocomial outbreaks worldwide with high-levels of antibiotic resistance including production of ESBLs, mainly CTX-M-15 (Han et al., 2021).

The *K. pneumoniae* ST3559 strain isolated from irrigation water in the current study were capsular type 27 and serotype O4, which is similar to an O4 serotype MDR *K. pneumoniae* outbreak strain from a neonatal care unit in sub-Saharan Africa (Cornick et al., 2020). In addition, *K. pneumoniae* ST3559 harboured the *bla*$_{\text{SHV-178}}$ gene which, to the best of our knowledge, have previously only been reported in clinical *Enterobacter hormaechei* strains from the First Affiliated Hospital of Zhejiang University in Hangzhou (Gou et al., 2020).

Apart from β-lactamase genes, the *K. pneumoniae* strains also harboured aminoglycoside, fosfomycin, fluoroquinolone, tetracyline, phenicol, trimethoprim and sulfonomide resistance genes, which is a greater diversity of resistance genes than previously reported in Enterobacteriaceae isolates from German surface waters (Falgenhauer et al., 2019). Similar to results of clinical *K. pneumoniae* strains reported by Mbelle et al. (2020) In191, harbouring *dfrA14* was identified in the three different *K. pneumoniae* sequence types of the current study, reiterating that it is not a narrow spectrum integron. In addition, *dfrA14b* was associated with *IS6* that has

previously been reported as having a vital role in the rearrangement and dissemination of antibiotic resistance (Varani et al., 2021). The presence of *fosA* and *sul2* in all the *K. pneumoniae* strains of the current study also correspond to the results reported by Mbelle et al. (2020) from clinical *K. pneumoniae* strains in Pretoria.

The high-level of trimethoprim resistance globally has however led to trimethoprim-sulfamethoxazole no longer being recommended for outpatient treatment of urinary tract infections and similarly, the use of fosfomycin might not be efficacious anymore (Mbelle et al., 2020). Four MDR *K. pneumoniae* isolates from irrigation water (ST15, n=1) and spinach (ST985, n=3) had O1 serotypes, previously reported as the most commonly isolated serotypes from human hosts and dominant in human disease (Follador et al., 2016). However, it is noteworthy that no genes encoding carbapenamases nor resistance to colistin were identified in the current study. All five characterised *K. pneumoniae* strains also harbored several virulence factors including those that coded for an iron uptake system (*kfu*) and type 3 fimbrial adhesins (*mrk*) that play an important role in adhesion to medical devices such as catheters (Albasha et al., 2020; Finton et al., 2020).

Serratia spp. are opportunistic pathogens that may pose a health threat to immunocompromised and hospitalised patients (Petersen and Tisa, 2013). The *S. marcescens* species is most often associated with nosocomial infections, however, *S. fonticola* has been reported to function as a human pathogen when detected alone or may be a bystander and act as carrier of resistance genes when discovered with other organisms (Petersen and Tisa, 2013; Aljorayid et al., 2016). Characterising virulence genes of the MDR environmental strains therefore becomes important within the plant-food producing environment. In the current study, all *S. fonticola* strains harboured $bla_{\text{SFO-1}}$ and numerous plasmid incompatibility (Inc) groups were identified in these *S. fonticola* strains (data not shown). However more in-depth plasmid typing and analysis will be required to fully understand the risk/probability of $bla_{\text{SFO-1}}$ dissemination in the environment where *S. fonticola* naturally occurs. In certain Enterobacteriaceae species, ESBL genes are inherently carried on chromosomes (Naas et al., 2008). This includes the $bla_{\text{SFO-1}}$ ESBL gene from *S. fonticola* that differs

from most class A ESBLs, as the β-lactamases' production can be induced by a high level of imipenem (Naas et al., 2008). The *bla*$_{SFO-1}$ ESBL does not form part of the most clinically relevant ESBLs and are therefore rarely reported.

Zhou et al. (2020) reported in contrast an increasing trend of the co-existence of plasmid-borne *bla*$_{SFO-1}$ and carbapenemase genes in clinical *Enterobacter* spp. in China. All the *S. fonticola* strains also harboured numerous fluoroquinolone resistance genes, raising a health concern for treatment options, as fluoroquinolones are often used for management of conditions including typhoid fever and MDR tuberculosis (Richards et al., 2019). Interestingly, one *S. fonticola* strain harboured an acquired trimethoprim (*sul2*) resistance gene associated with IS110, corresponding to *K. pneumoniae* from a German university hospital (Schwanbeck et al., 2021).

The *Serratia* genus naturally lacks resistance genes for trimethoprim and sulfonamides (Sandner-Miranda et al., 2018). Previous reports of potential pathogenic *S. fonticola* primarily focused on the antibiotic resistance profiles (Tasić et al., 2013; Aljorayid et al., 2016; Hai et al., 2020). The strains from the current study additionally harboured various virulence factors. This included flagellar biosynthesis- and chemotaxis-related genes as well as genes encoding iron uptake systems corresponding to those previously reported in important MDR nosocomial pathogenic *S. marcescens* (Iguchi et al., 2014).

Only one *S. enterica* strain isolated from river irrigation water was characterised in the current study. Irrigation water is well documented as a source for fresh produce contamination of foodborne pathogens including *Salmonella* spp. (Liu et al., 2018). The strain harboured an AmpC resistance gene, similar to *S. enterica* characterised from surface water in the United States (Li et al., 2014). In addition, the *S. enterica* from the current study carried aminoglycoside resistance genes (*aac(6')-Iaa* and *aac(6')-Iy*), similar to results reported by Nair et al. (2016) for non-typhoidal *Salmonella* spp. isolated from a United Kingdom population. Of the 23 known *Salmonella* SPIs previously described (Mansour et al., 2020), the isolate from the current study carried 11 SPIs. This included

SPIs that are commonly reported in *S. enterica* and encode genes responsible for enabling invasion of epithelial cells (SPI1), facilitating the replication of intracellular bacteria (SPI2), adhesion to epithelial cells (SPI3, 4, 5, and 9) (Waterman and Holden, 2003; Velásquez et al., 2016; Mansour et al., 2020), as well as SPI13 and 14 which corresponds to being part of the core genome of invasive non-typhoidal *Salmonella* spp. (Suez et al., 2013). Additionally, pathogenicity islands C63PI and CS54 were present in the *S. enterica* strain in this study, which has previously been found in the S. Typhimurium and S. Typhi genomes (Sabbagh et al., 2010; Jibril et al., 2021). Since no phenotypic indication of virulence was investigated, the prediction of virulence genes using *in silico* tools should be regarded with care, however, using PathogenFinder, the *S. enterica* strain from the current study showed 94% probability of being a human pathogen.

7.5 Conclusion

This is the first WGS analysis study of MDR ESBL/AmpC-producing *E. coli*, *K. pneumoniae*, *S. fonticola* and *S. enterica* isolates from spinach production systems within SA. The selected isolates represent potential pathogenic genera listed by the WHO as a priority for surveillance of antimicrobial resistance screening. Numerous clinically relevant resistance genes were detected in the screened samples. This study showed the potential of using WGS in metadata studies for detailed molecular characterization of potential pathogenic Enterobacteriaceae. Furthermore, the study highlighted the importance of the agricultural production environment as a source of antibiotic resistance genes within Enterobacteriaceae in the water-plant-food interface. The results from this study highlights the need for expanded surveillance in agricultural systems. Future studies should include a more in-depth and controlled analysis, with a greater number of sequenced isolates from the farm-to-retail to better understand the prevalence of resistance gene transmission through the supply chain.

7.6 References

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Chapter 8

"The important thing is not to stop questioning. Curiosity has its own reason for existence. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery each day."
-Albert Einstein

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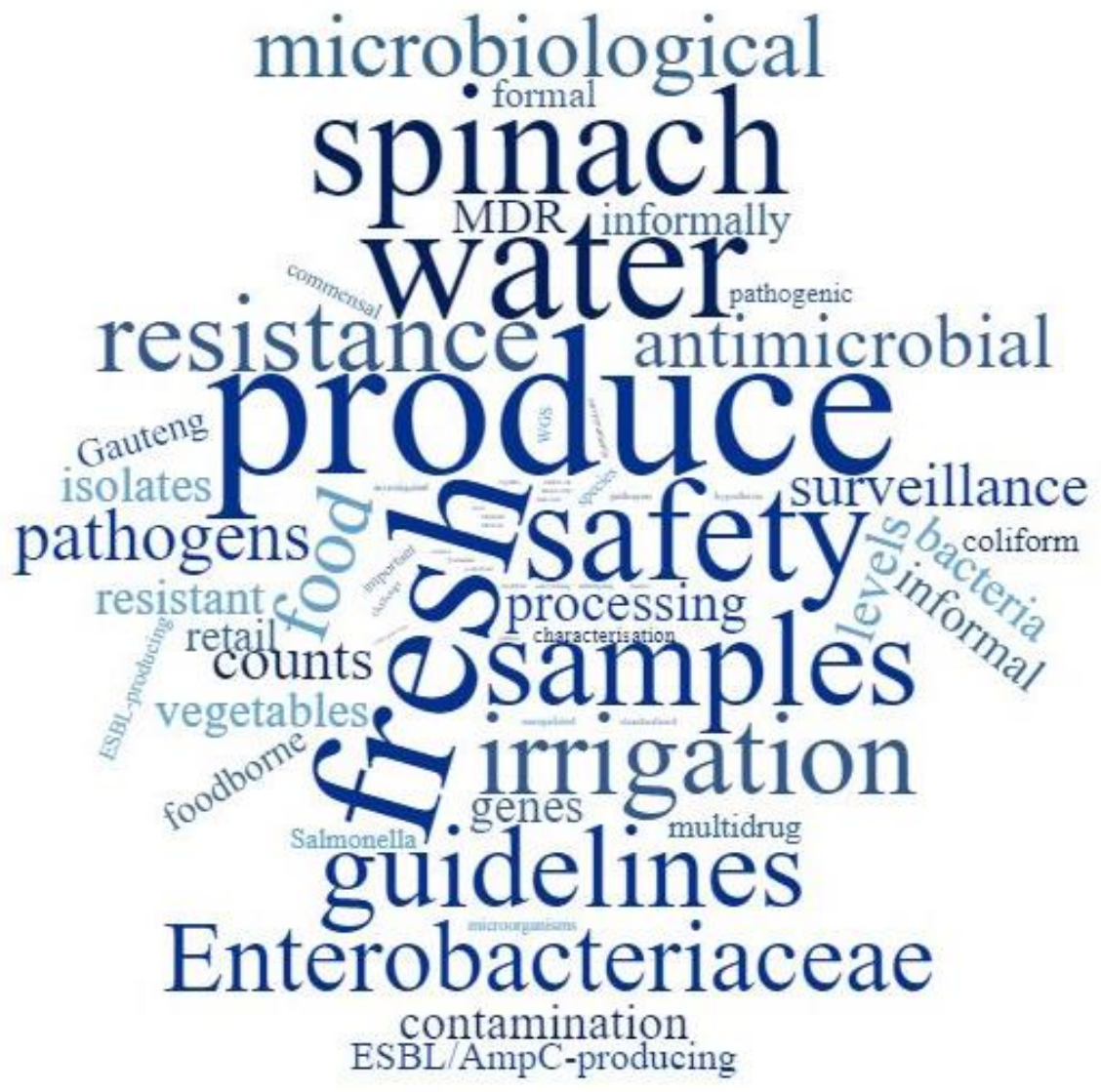

General Discussion

Consumption of fresh produce is vital for a healthy diet and a strong immune defence system. With increased consumption comes greater risks in the food system. Fresh produce safety is thus a global priority and requires improved production systems from the farm to the consumer. Understanding the microbiological quality of fresh fruit and vegetables are important as it directly relates to safety of fresh produce (Schuh et al., 2020). In this thesis the microbiological safety of commonly consumed raw vegetables was studied. The focus was on occurrence and characterisation of potential human pathogens with expanded antimicrobial resistance from fresh produce retailed formally and informally and particularly the commercial leafy greens supply chain. Commercial spinach supply chains were monitored from the farm, through processing up to retail in Gauteng, the most densely populated province in SA. The study included a multi-perspective approach in microbiological food safety with a focus on traditional indicator bacteria (*Escherichia coli*) and foodborne pathogens (*E. coli*, *Salmonella* spp., and *Listeria monocytogenes*) as well as antimicrobial resistance phenotypic and genotypic characterisation of Enterobacteriaceae. Three main hypotheses were investigated as described in Chapter 1 and will be assessed in this final concluding section of the thesis.

Hypothesis 1: Occurrence of antimicrobial resistant Enterobacteriaceae is higher and microbiological safety parameters unsatisfactory for fresh produce sold in the informal compared to formal markets.

Fresh produce safety at the point of sale

The objectives of the scoping study of 545 fresh produce samples at the point of sale (Chapter 3 and Chapter 4) included microbiological safety analysis (coliforms, *E. coli* and Enterobacteriaceae counts), detection and characterization of potential foodborne pathogens (*E. coli*, *Salmonella* and *Listeria monocytogenes*) as well as isolation and characterization of

extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae (Richter et al., 2019, 2021). The microbiological safety of fresh produce at retail has been studied globally with the focus mainly on assessing indicator bacteria levels and detection and characterisation of foodborne pathogens (*E. coli*, *Salmonella* spp., and *L. monocytogenes*) (Vital et al., 2014; Denis et al., 2016b; du Plessis et al., 2017; Li et al., 2017; Roth et al., 2018a). The lack of consensus in guidelines with regard to acceptable hygiene indicator bacteria levels on ready-to-eat (RTE) fresh produce renders compliance according to different countries difficult. Moreover, current national recommendations are needed for SA. These could include adoption of established and tested recommendations, such as those stipulated by the European Union, adjusted accordingly to be country specific.

In the South African context, adding complexity to integrated fresh produce safety and antimicrobial resistance surveillance in plant-based agriculture, is the dualistic fresh produce food supply system. Both commercial and small-scale farmers supply fresh produce to the public, with distribution channels that go through a formal (regulated) or an informal (unregulated) system. To date, limited information is available regarding the microbiological safety and prevalence of antimicrobial resistance and virulence genes in bacterial isolates from fresh produce sold informally compared to that from formal retailers in SA. Moreover, no studies have investigated the presence of multidrug resistant ESBL-producing potential pathogens in fresh produce sold in the different SA trading sectors. Yet, 50% of the local population depend on informal trade (Petersen and Charman, 2018).

The results from Chapter 3 showed that coliforms, *E. coli* and Enterobacteriaceae enumerated from produce retailed formally and informally were mostly not significantly different, with some exceptions noted. An overall statement could therefore not be made regarding the

microbiological safety of fresh produce sold informally compared to that from formal retailers. Per product type, coliform counts that were not significantly different in the current study from the formal and informal markets corresponded to Du Plessis et al. (2017) who reported no significant difference in coliform counts on spinach from informal vendors and formal retailers in Gauteng Province.

As coliforms form part of the natural microflora of fruit and vegetables, testing for total coliforms in fresh produce food safety practices is not intended to detect faecal contamination, but rather to reflect general hygiene during food production or handling [Centre for Food Safety (CFS), 2014]. For this reason, enumeration of Enterobacteriaceae, which includes a larger selection of potential pathogens than coliforms (Baylis et al., 2011), might give a better reflection of the microbiological safety and possible mitigation necessary to minimize bacterial contamination. However, most international guidelines omit the coliforms and Enterobacteriaceae criteria completely for fresh fruit and vegetables due to the natural high bacterial load on these products (Health Protection Agency, 2009; Health Canada, 2010; FSAI, 2016; FPSC A-NZ, 2019). The main hygiene indicator used in fresh produce safety being *E. coli*, with varied acceptable limits in different countries.

Acceptable *E. coli* limits for retailed fresh produce differ with guidelines specified in the UK as 20 to 100 CFU/g, Australia as 3 to 100 CFU/g, and Canada as 100 MPN/g, while the SA Department of Health (DoH) guidelines which are currently under revision proposed zero *E. coli* per gram for raw fruit and vegetables (DoH, 2000; FSANZ, 2001; Health Protection Agency, 2009; Health Canada, 2010). The results from Chapter 3 showed that 44% of the spinach samples from farmers' markets harboured *E. coli* with significantly higher mean *E. coli* counts (1.22 log CFU/g) than the 12% of spinach samples from street traders that harboured *E. coli*. However, no significant difference in the mean *E. coli* counts from the street

traders (0.25 log CFU/g) and 28% trolley vendor spinach samples positive for *E. coli* (0.72 log CFU/g) were seen compared to the 20% retailer spinach samples that harboured *E. coli* with a mean value of 0.84 log CFU/g. This contrasts a previous study where *E. coli* counts on spinach purchased from informal vendors were significantly higher than that of spinach from formal retailers (du Plessis et al., 2017). Interestingly, Baloyi et al. (2021) enumerated *E. coli* from only 2% of tomato samples (n=50) purchased from informal markets in Gauteng Province, while the current scoping study reflected higher *E. coli* occurrence (73%) in tomatoes retailed informally. In 20% of the tomato samples from the farmers' markets (n=50), *E. coli* were enumerated at levels that were not significantly different than that of the 94%, 100% and 98% retailer (n=50), street trader (n=50) and trolley vendor (n=50) tomato samples that also harboured *E. coli*, respectively. However, after enrichment, only tomato samples from farmers' markets in the current study were positive for *E. coli* isolates, while Baloyi et al. (2021) isolated *E. coli* from informally street vended tomatoes in Gauteng Province.

In addition to assessing hygiene indicator bacteria levels and foodborne pathogen presence in fresh produce, inclusion of surveillance of antimicrobial resistance and the genetic determinants from bacteria found on fresh produce in food safety research has become more common (Ben Said et al., 2016; Hölzel et al., 2018). Overall, 81/545 samples (14.86%) harboured *E. coli* in the current study, the majority isolated from farmers' market produce samples. Of the 67 characterised isolates, 40.3% were multidrug resistant (MDR) (Richter et al., 2021, Chapter 3). This is similar to the 37.9% multidrug resistance reported in *E. coli* (n=29) isolated from spinach retailed formally and informally in Gauteng (du Plessis et al., 2017), but lower than the 85.7% multidrug resistance in *E. coli* (n=48) isolated from spinach, tomatoes, carrots, cabbage and apples from Gauteng informal markets (Baloyi et al., 2021).

Antimicrobial resistance genes in addition to acquisition of virulence genes increases the pathogenicity of microorganisms and consequently the severity of infection (El-Baky et al., 2020). Commensal bacteria may act as a reservoir for transferring resistance genes to pathogens (Hassan et al., 2011; Al-Kharousi et al., 2019). Consequently, enumeration of Enterobacteriaceae and associated antimicrobial resistance genes have been considered as an additional microbiological safety parameter in food supply, to include a better indication of both commensal and potential pathogenic bacteria levels on fresh produce, (Al-Kharousi et al., 2016; Liu and Kilonzo-Nthenge, 2017). Although MDR *E. coli* was not isolated from samples from all the different vendors, ESBL-producing Enterobacteriaceae were isolated from 95/545 (17.43%) of the samples that included produce from all vendors (Chapter 4). This is higher than the 13.3%, 5.5%, and 0.83% ESBL-producing Enterobacteriaceae occurrence reported from retailed fresh produce in similar studies within the same sampling period in China, Romania and South Korea, respectively (Ye et al., 2017; Colosi et al., 2020; Song et al., 2020).

Dissemination of antimicrobial resistant organisms globally is a major public health challenge, threatening effective prevention and treatment of an increased amount of bacterial infections (Prestinaci et al., 2015; Vikesland et al., 2019). Moreover, ESBL-producing Enterobacteriaceae forms part of the global priority list of antibiotic resistant bacteria as these pathogens cause high morbidity and mortality and increased healthcare costs (WHO, 2017).

Enterobacteriaceae regarded as emerging bacterial threats include *E. coli*, *K. pneumoniae* and *Enterobacter* spp. showing resistance to β-lactams and aminoglycosides (Fair and Tor, 2014). Dominant ESBL-producing Enterobacteriaceae identified in the current study were *E. coli*, *Enterobacter cloacae, Enterobacter asburiae* and *K. pneumoniae* (Chapter 4). From spinach and tomato samples, which were the two products sampled from all the different vendors, the highest number of ESBL-producing isolates were from samples purchased from street traders,

followed by retailers, trolley vendors and farmers' markets. In contrast, the majority MDR generic *E. coli* isolates were from farmers' market spinach and tomato samples, followed by samples from street traders and retailers, while no MDR generic *E. coli* was isolated from the spinach and tomato samples bought from the mobile trolley vendors.

Additional products (lettuce, cucumber and green beans) were analysed from farmers' markets as no South African farmers' market fresh produce microbiological safety data exists to date. Naicker and Rogerson (2017), recently highlighted the growing expansion of farmers markets in SA as part of a wider trajectory of local and alternative food networks. Similar to results reported by Colosi et al. (2020), the fresh produce analysed from the farmers' markets in the current study also harboured ESBL genetic determinants. The occurrence of MDR *E. coli* and more notably ESBL-producing Enterobacteriaceae reported for the first time in formally and informally retailed fresh produce in SA highlights the importance of expanded routine investigations of environmental bacteria. This is necessary as predictive data on the development of antimicrobial resistance in the environment will contribute towards mitigation strategies of antimicrobial resistance within the one health framework (WHO, 2017).

Conclusions based on the analysis of the fresh produce at the point-of-sale scoping study include: i) MDR ESBL/AmpC-producing potential pathogenic Enterobacteriaceae are present in raw vegetables retailed formally and informally at selected sites in Gauteng with no definitive difference in occurrence between produce from the different trading sectors; ii) Expanded microbiological safety surveillance for retailed fresh produce is necessary in different SA provinces, especially within the currently unregulated informal fresh produce trade, that supplies to a large proportion of the SA population; iii) The occurrence of MDR potential human pathogens and MDR commensal *E. coli* in retailed fresh produce highlights the need for standardized commodity specific fresh produce safety guidelines, with inclusion

of antimicrobial resistance surveillance in food safety strategies; iv) Improved antimicrobial resistance surveillance is necessary in fresh produce production systems from farm-to-retail, to identify potential sources of contamination, as ten different genera of ESBL/AmpC-producing Enterobacteriaceae, including clinically significant species, were isolated from the retailed fresh produce.

Hypothesis 2: Microbiological quality of irrigation water contributes towards the presence and persistence of antimicrobial-resistant bacteria in the spinach production system.

Significance of irrigation water microbiological quality in fresh produce production

As fresh produce is produced in a natural environment, the natural occurrence of microorganisms on fruit or vegetables is expected (Berger et al., 2010; Beharielal et al., 2018). Accordingly, Enterobacteriaceae was enumerated from spinach samples at various stages of production and processing as well as spinach samples at retail, regardless of the source of irrigation water used (Chapter 5). The results from the current study confirmed the second hypothesis as a link between the *E. coli* isolated from the irrigation water and spinach at harvest, through processing and at retail was shown and the irrigation water quality dictated the potential of pathogen contamination in fresh produce production.

International guidelines and regulations for agricultural water quality vary by country/region (Banach and Van Der Fels-Klerx, 2020), while fresh produce industries such as the Leafy Greens Marketing Agreement (LGMA) (https://lgma.ca.gov/food-safety-progra–m) in the U.S. has commodity specific guidelines for production and harvest of lettuce and leafy greens. The guidelines are often based on the U.S. Food Safety Modernisation Act (FSMA) with a strong food safety focus shifting from responding to preventing foodborne illness (FDA, 2021). These guidance documents stipulate different acceptable *E. coli* levels based on the risk of types of agricultural water systems and specific uses within production and processing of leafy greens

(https://lgma.ca.gov/food-safety-program), while other guidelines include both coliforms and *E. coli* limits as criteria for potential contamination. More specifically, the LGMA and produce safety rule of the FSMA propose a water microbiological quality standard of average generic *E. coli* levels <126 MPN/100ml for multiple samples of irrigation water used in leafy green production (Haymaker et al., 2019).

According to the World Health Organisation (WHO) irrigation water quality recommendations, fecal coliform levels in irrigation water used for minimally processed fresh produce should not exceed 1000 CFU/100 ml (WHO, 2006). Similarly, the Department of Water Affairs (DWAF) guidelines in SA stipulate that water used for vegetable and crop irrigation should have coliform levels <1000 CFU/100 ml and that there is likelihood of contamination of vegetables and other crops eaten raw if the *E. coli* counts range between 1-1000 CFU/100 ml (DWAF, 1996). The coliform counts of the river water and borehole water from the storage dam in the current study exceeded these recommendations. The irrigation water from the pivot point that came in contact with the harvested spinach for two of the three farms in the current study would also not have been acceptable according to the DWAF (1996) guidelines, as the river irrigation water had mean *E. coli* counts of 2.02 log MPN/100ml and the irrigation water of Farm B after circulation in the second storage dam had mean *E. coli* levels of 2.62 log MPN/100ml. Although enumeration of *E. coli* is routinely used as an indicator of fecal contamination in water sources used in fresh produce production worldwide, no standardized global guidelines exist. Moreover, the extraordinarily high pathogenic loads present in South African surface water often used for agricultural irrigation purposes poses a particular challenge for fresh produce safety.

Recently, a joint Food and Agriculture Organization (FAO) and WHO report stated that the assessment of *E.coli* levels alone in irrigation water for safe use in food safety is not an appropriate measure as it is not considered a proper surrogate for the diversity of potential

pathogens that may be present (FAO and WHO, 2019). Moreover, the presence of generic (non-pathogenic) *E. coli* are reported as poor indicators of the presence of STEC (Haymaker et al., 2019). Further to this, the results from Chapter 3 and Chapter 4 of fresh produce at the point of sale also indicated that determining the presence of *E. coli* levels alone is not a good indicator of prevalence of antimicrobial resistance genes.

The LGMA commodity specific irrigation water guidelines recommends the inclusion of STEC (including *E. coli* O157:H7) and *Salmonella* in follow-up water testing if the overhead irrigated leafy greens had direct contact with irrigation water exceeding the specific *E. coli* acceptance criteria (https://lgma.ca.gov/food-safety-program). No specific South African guidelines exist, nor guidelines in many other countries, for the presence of *Salmonella* spp. or other pathogens in irrigation water, which might result in underreporting. Expanded irrigation water guidelines with inclusion of a wider range of pathogens should therefore be considered. However, regional challenges in SA and other developing countries should also be considered as expanded monitoring and implementation might not always be realistic.

Previous studies have shown that multidrug resistant Enterobacteriaceae, including commensal and potential human pathogenic isolates are present in South African irrigation water sources and commercially produced leafy greens (Njage and Buys, 2014; Jongman and Korsten, 2016a). After enrichment in the current study, generic *E. coli* was isolated from 40.30 % and 14.60 % of water and spinach samples, respectively (Chapter 5). Collectively, 43.73% (n=80) were MDR and ERIC-PCR cluster analysis showed that *E. coli* isolates from irrigation water and spinach at retail within each respective supply chain had at least 85% similarity. Concomitantly, ESBL/AmpC-producing Enterobacteriaceae were isolated from 29.1% and 37.5% spinach samples from the respective production scenarios and 20.83% river and borehole (10.42%) water (Richter et al., 2020; Chapter 6).

Interestingly, a greater abundance and species diversity from harvested, to processed- and subsequent retail spinach samples were seen throughout the chains. Isolates from retailer spinach samples included *K. pneumoniae, S. fonticola, R. aquatilis, E. coli* and *E. asburiae* that corresponded to isolates from retailed fresh produce samples in similar studies (Ye et al., 2017a; Zekar et al., 2017), as well as ESBL-producing Enterobacteriaceae isolated from formally and informally retailed fresh produce as reported in Chapter 4 (Richter et al., 2019). The results from Chapter 5 and Chapter 6 reiterates the contribution of irrigation water as a source of antimicrobial resistant bacterial contamination in fresh produce production as previously reported (Vital et al., 2018). Yet, relevant, standardized data for elucidating the role of plant-based agriculture in the holistic picture of AMR ecology is still lacking globally (FAO, 2018).

Analysis of the the spinach supply chains confirmed the second hypothesis and conclusions include: i) a high prevalence of multidrug resistance in commensal and potential pathogenic Enterobacteriaceae isolated from contaminated river and borehole irrigation water and associated spinach at harvest, throughout processing and at retail; ii) resistance genes persist throughout processing of fresh produce in both washed and unwashed commercial spinach product lines as ESBL/AmpC-producing MDR organisms with similar phenotypic AMR profiles were isolated from harvested spinach, spinach during processing and samples from the retailer; iii) where contaminated river water with unacceptable microbiological quality according to the current guidelines were used for irrigation, *E. coli* was enumerated from spinach samples throughout the complete chain and an increase in ESBL/AmpC-producing Enterobacteriaceae genera were seen in isolates from the spinach samples; iv) the microbiological quality of the initial source water played a vital role in the retailed fresh produce microbiological quality; v) there is a need for standardized risk-based water quality

guidelines for fresh produce production where the context of water uses along the food chain has to be considered in a fit-for-purpose manner.

Hypothesis 3: Clinically relevant antibiotic resistance genes are present in Enterobacteriaceae isolated from commercial spinach production environments.

Food safety, antimicrobial resistance and one health

This hypothesis was cofirmed with WGS analysis of selected isolates from the spinach supply chains. Application of WGS is increasingly used for surveillance within food supply chains. This follows as a single assay can provide information regarding antimicrobial resistance, mobile genetic elements (e.g. insertion sequences, plasmids and integrons), serotypes and determining the pathogenicity. All these factors are crucial in defining and attributing infection sources of food-related outbreaks caused by antimicrobial resistant microorganisms (Oniciuc et al., 2018b). Although clinically relevant bacteria were isolated from the water and spinach sources and antibiotic resistance genes associated with previous outbreak strains were detected, it is noteworthy that no genes encoding carbapenamases nor resistance to colistin were identified in the current study. This study was the first to report on WGS characterisation of MDR ESBL/AmpC-producing Enterobacteriaceae from fresh produce supply chains in SA (Chapter 7). Globally, limited quantitative data is available and a lack of understanding regarding the behaviour and persistence of microbial hazards introduced via irrigation water, and the interaction of water with different fresh produce products in varied environments at different steps along the supply chain remains (FAO and WHO, 2019). Only once sufficient data is generated, risk assessments for AMR within fresh produce supply chains can be initiated.

The overuse and misuse of antibiotics is a worldwide problem and this thesis outlined that resistance to antibiotics also forms part of food safety challenges within South African fresh produce supply. Recently, the global COVID-19 pandemic highlighted how crucial

surveillance systems are for detection and management of public health threats. If antimicrobial resistance is not tackled aggressively, this emerging threat will also lead to permanent humanitarian and economic consequences globally. However, to establish effective surveillance programs, standardised data acquistion and analysis is required.

The overall results from this study showed that traditional microbiologial methods still have a very important role in food safety strategies, albeit fresh produce and irrigation water microbiological quality guidelines need to be re-assessed and standardised. Moreover, government guidelines for fresh produce are currently absent in SA. Additional inclusion of molecular techniques such as WGS within these food safety strategies provides a myriad of information through which bacterial isolates from environmental and clinical settings can easily be linked, which is crucial for foodborne outbreak investigations and surveillance systems. Furthermore, mitigation strategies and improved food safety surveillance and awareness training is required especially in the unregulated informal sector that play a vital role in food supply for the SA population. This follows as a high prevalence of MDR ESBL/AmpC-producing Enterobacteriaceae that included clinically significant species were isolated from the informally traded raw fresh produce. No- or very limited tracking systems currently exist regarding the source of fresh produce retailed informally. Furthermore, the microbiological quality of associated irrigation water used during production as well as the wash water used on site at the different street traders, in which the fresh produce was continously soaked, remains unknown. Analysis within the formal commercial spinach supply chains, where sufficient source-tracking systems are in place, emphasised the important role that the water quality plays during production and processing for the final retailed product and further, the need for surveillance of antimicrobial resistance within the water-plant-food-human health interface.

On a final note, the occurrence of MDR potential pathogenic Enterobacteriaceae with expanded resistance profiles were reported for the first time in fresh vegetables sold formally and informally as well as water sources and irrigated spinach from commercial production systems in SA. Worldwide the dangers of AMR have been known for years, yet the extent, emergence and maintenance of MDR organisms in plant production remain underreported within the one health context of combatting AMR. From a traditional food safety perspective, this thesis presents evidence that a pardigm shift in microbiological quality parameters, which currently focuses on hygiene indicator microorganisms (fecal coliforms, *E. coli*) in the SA water-plant-food interface, is needed for a holistic microbiological safety profile of fresh produce to be consumed raw. Inclusion of additional members of Enterobacteriaceae often implicated in foodborne disease outbreaks (i.e. *Salmonella* spp.), other microorganisms such as protozoa and viruses as well as surveillance of AMR needs to be considered.

Training and awareness of responsible application of antimicrobials in agriculture, consequences of misuse, and the severity of the problem in the food chain in both formal and informal fresh produce production systems need to be improved. Moreover, this study showed that a national database of AMR surveillance within the water-plant-food-human health nexus needs to be established as this information is essential for future development and implemenation of risk mitigation strategies. Through inclusion of WGS analysis in food safety surveillance, a global link between potential pathogens and AMR gene dissemination can be established. Antibiotic resistance is a known major global health threat, exacerbated by the growing demand in food supply and recent increased use of antibiotics in response to the COVID-19 pandemic. Resistance gene dissemination among microorganisms has no regard for borders and continents, therefore, a global collaborative multisectoral approach to detect, prevent, and respond is vital.

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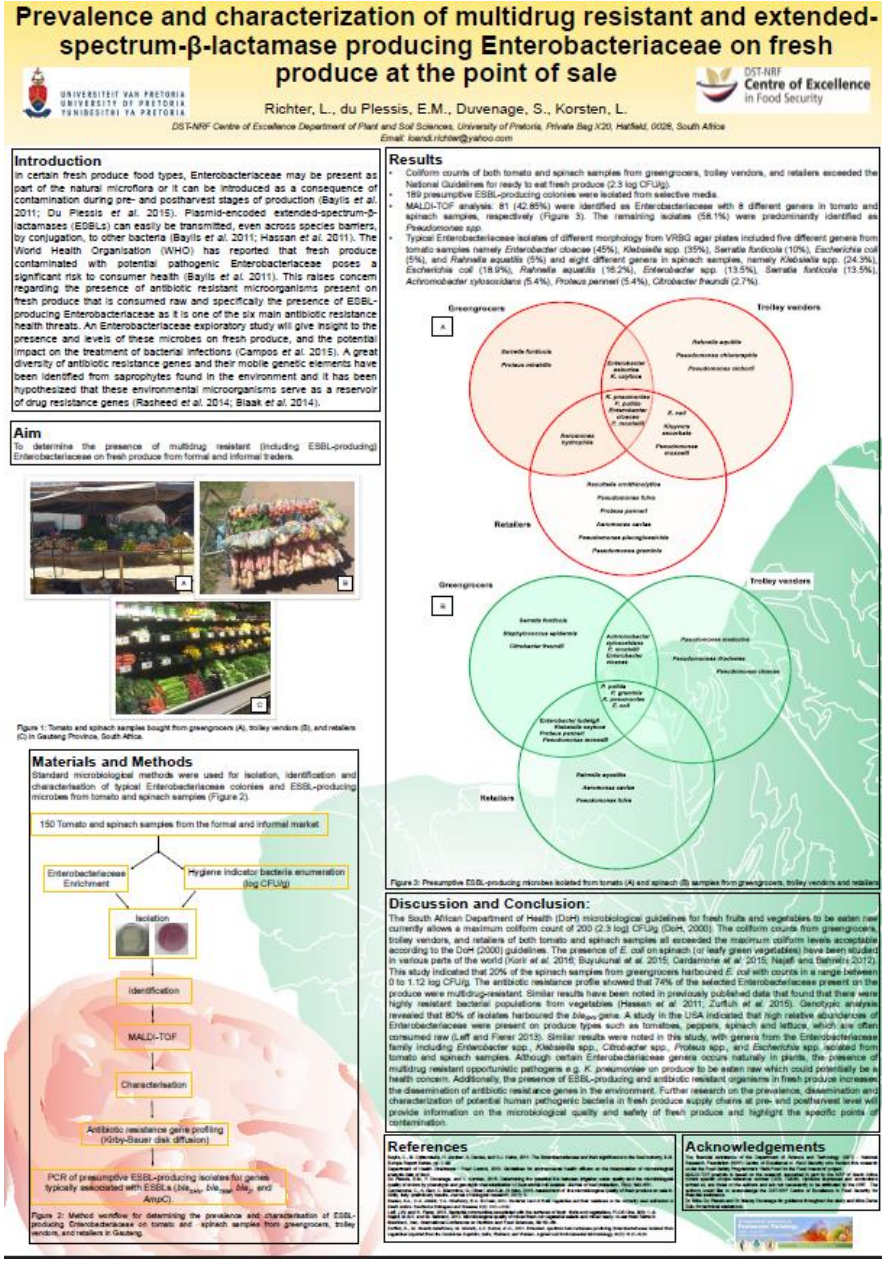

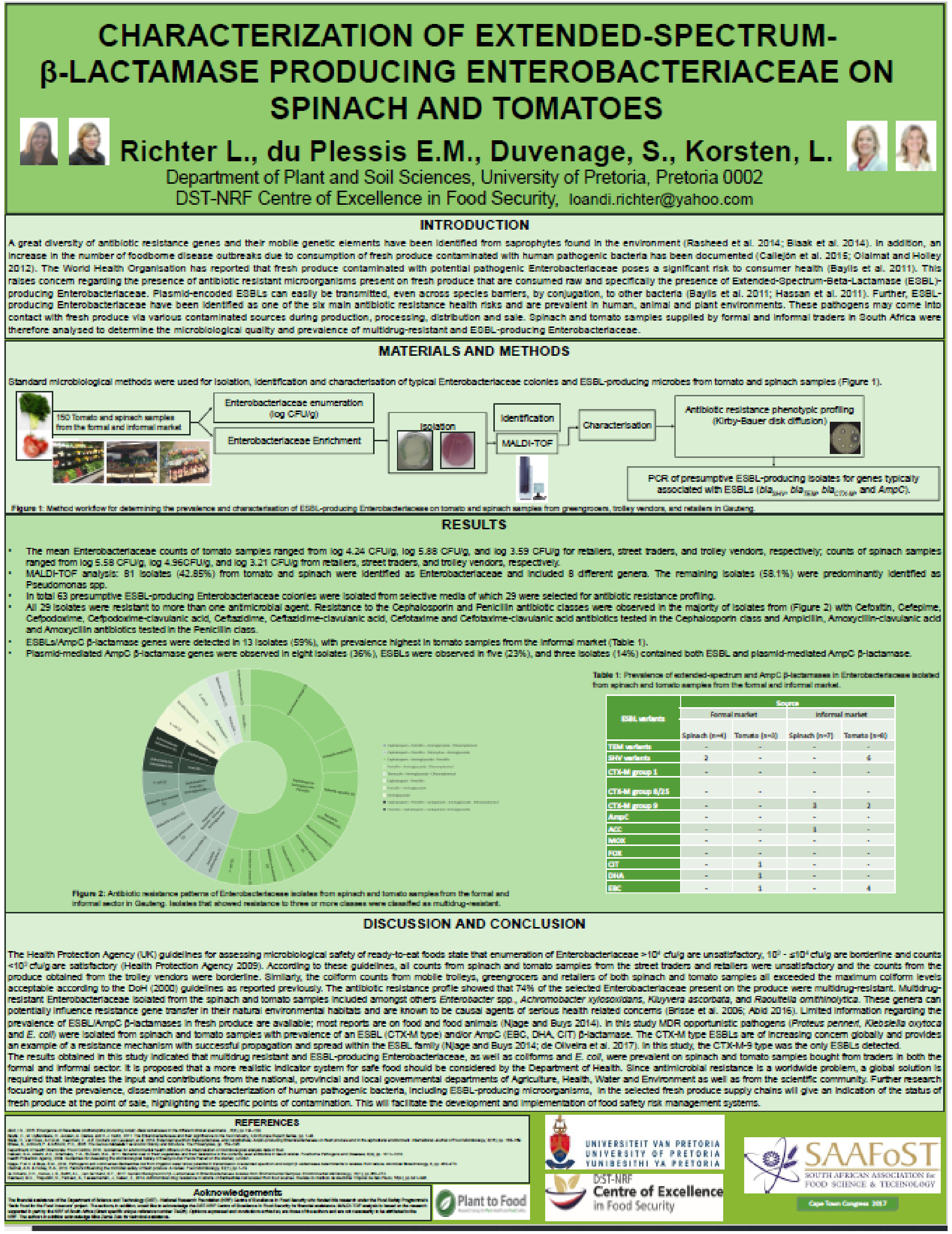

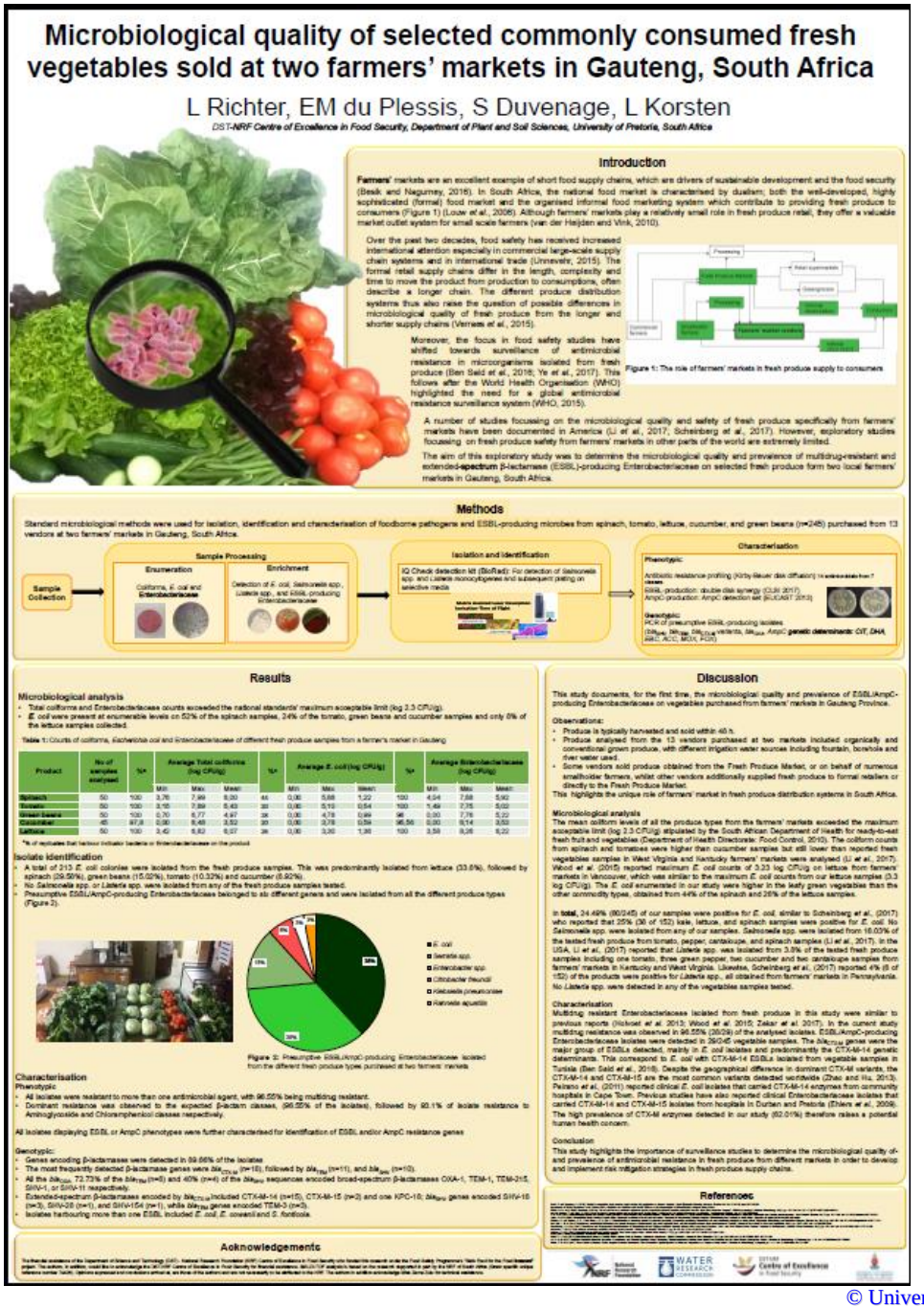

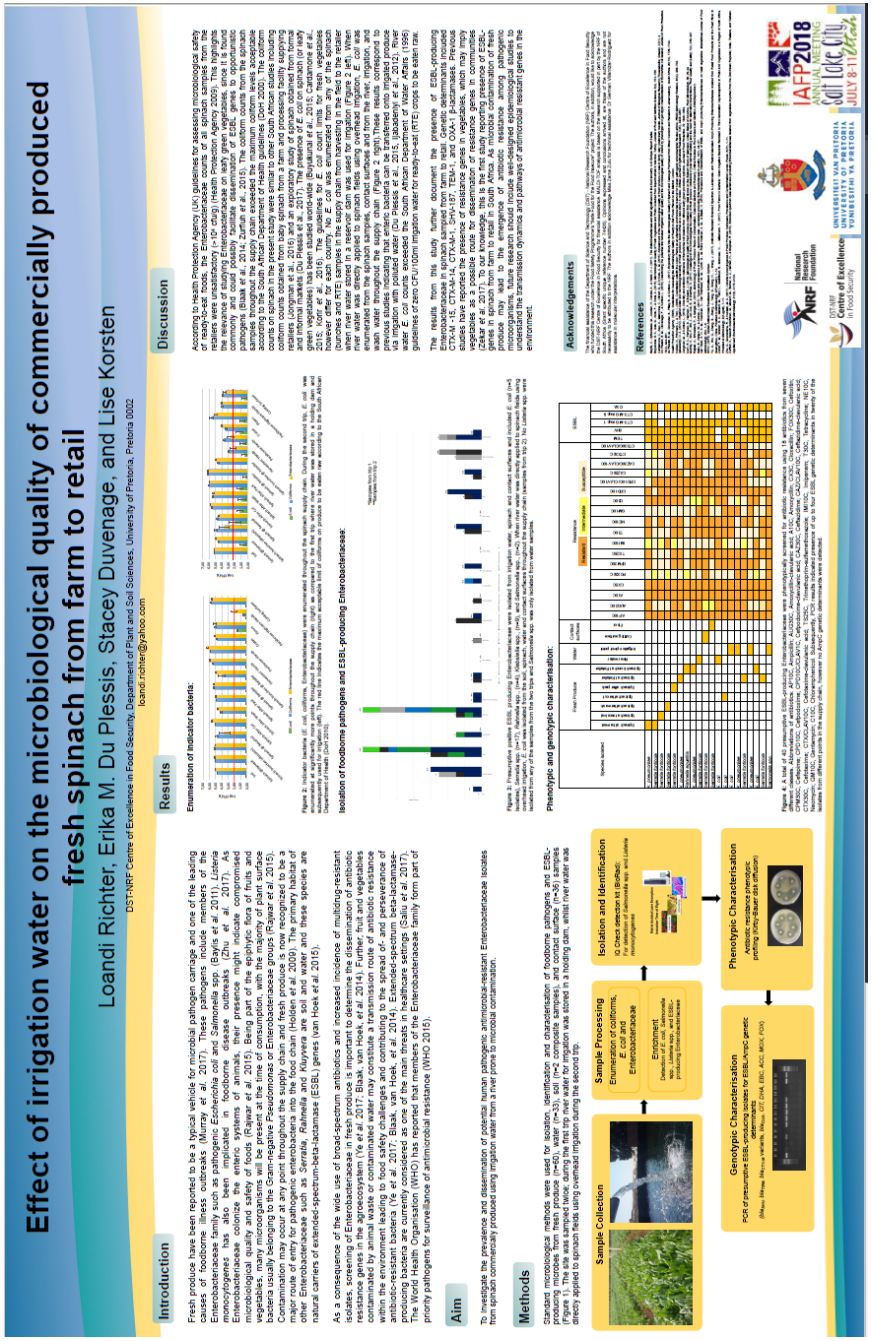

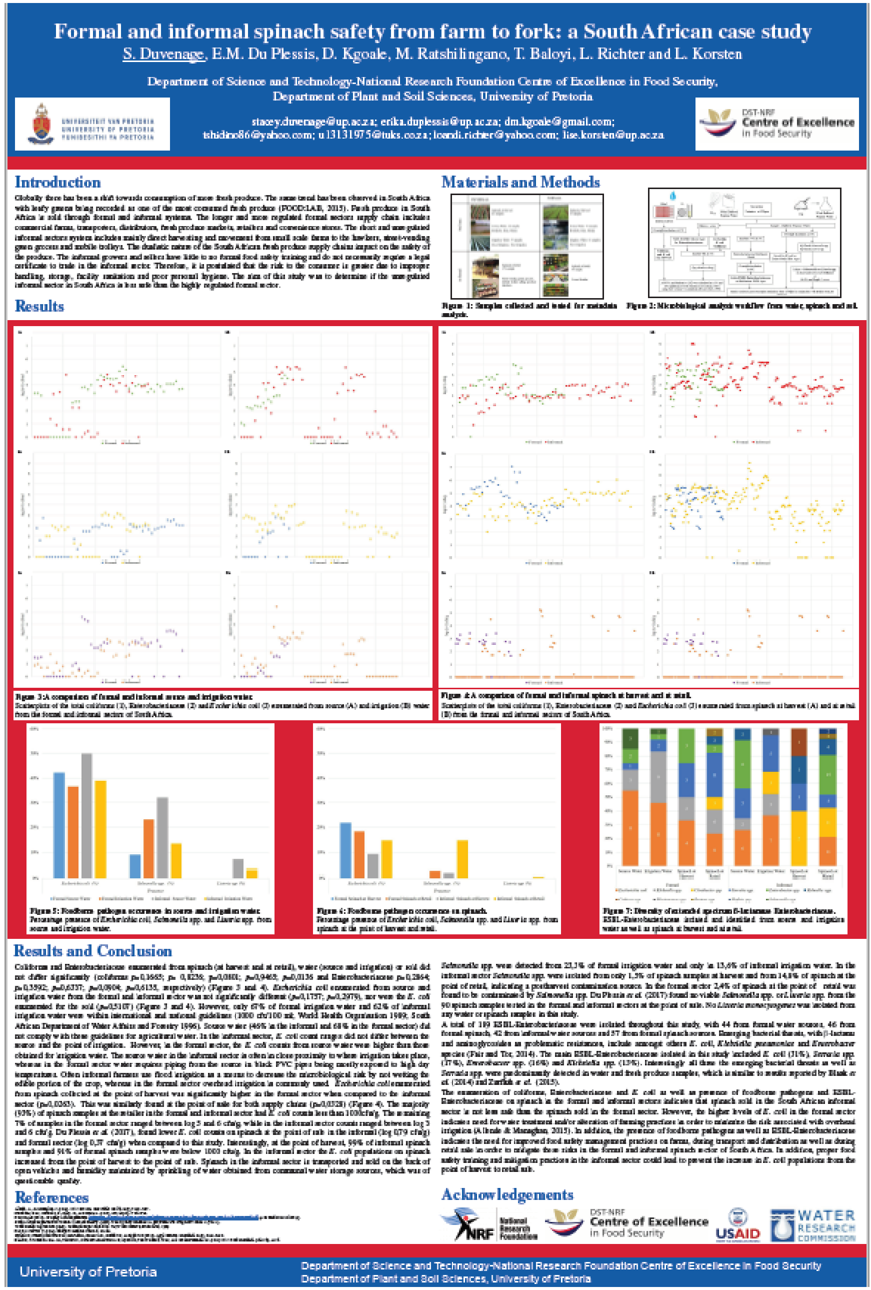

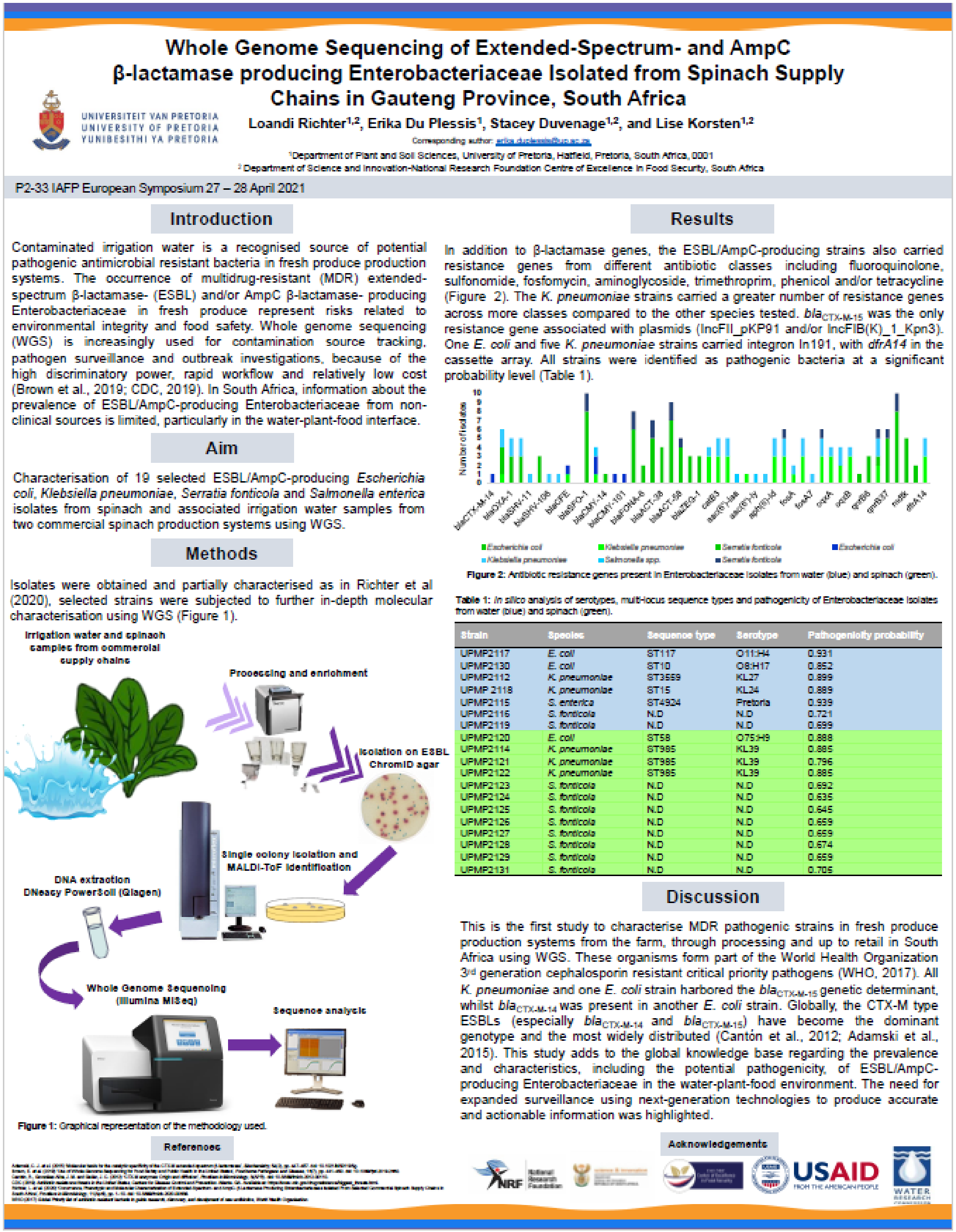

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Occurrence, Identification, and Antimicrobial Resistance Profiles of Extended-Spectrum and AmpC β-Lactamase-Producing *Enterobacteriaceae* from Fresh Vegetables Retailed in Gauteng Province, South Africa

Loandi Richter, Erika M. Du Plessis, Stacey Duvenage, and Lise Korsten

Abstract

Extended-spectrum β-lactamase (ESBL) and AmpC β-lactamase-producing *Enterobacteriaceae* are no longer restricted to the health care system, but represent increased risks related to environmental integrity and food safety. Fresh produce has been increasingly reported to constitute a reservoir of multidrug-resistant (MDR) potential human pathogenic *Enterobacteriaceae*. This study aimed to detect, identify, and characterize the antimicrobial resistance of ESBL/AmpC-producing *Enterobacteriaceae* isolates from fresh vegetables at point of sale. Vegetable samples (spinach, tomatoes, lettuce, cucumber, and green beans; $n = 545$) were purchased from retailers in Gauteng, the most densely populated province in South Africa. These included street vendors, trolley vendors, farmers' market stalls, and supermarket chain stores. Selective enrichment, plating onto chromogenic media, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) confirmation of isolate identities showed that 17.4% (95/545) vegetable samples analyzed were contaminated with presumptive ESBL/AmpC-producing *Enterobacteriaceae*. Dominant species identified included *Escherichia coli*, *Enterobacter cloacae*, *Enterobacter asburiae*, and *Klebsiella pneumoniae*. Phenotypic antibiotic resistance analysis showed that 96.1% of 77 selected isolates were MDR, while resistance to aminoglycoside (94.8%), chloramphenicol (85.7%), and tetracycline (53.2%) antibiotic classes was most prevalent. Positive phenotypic analysis for ESBL production was shown in 61 (79.2%) of the 77 isolates, and AmpC production in 41.6% of the isolates. PCR and sequencing confirmed the presence of β-lactamase genes in 75.3% isolates from all vegetable types analyzed, mainly in *E. coli*, *Enterobacter* spp., and *Serratia* spp. isolates. CTX-M group 9 (32.8%) was the dominant ESBL type, while EBC (24.1%) was the most prevalent plasmidic type AmpC β-lactamase. Our findings document for the first time the presence of MDR ESBL/AmpC-producing *Enterobacteriaceae* in raw vegetables sold at selected retailers in Gauteng Province, South Africa.

Keywords: antibiotic resistance, fresh produce, food safety

Introduction

Extended-spectrum β-lactamase (ESBL)- and AmpC-producing *Enterobacteriaceae* have increased in occurrence globally in health care systems, agroecosystems, and fresh produce, due to the widespread use of broad-spectrum antibiotics (Ye *et al.*, 2017). Dissemination of these antimicrobial-resistant microorganisms has been identified as one of the six main antibiotic resistance (AR)–related health risks globally (WHO, 2015). If infection by ESBL/AmpC-producing *Enterobacteriaceae* occurs, treatment options become limited as a result of expanded AR of the corresponding isolates (Freitag *et al.*, 2018). Since ESBL/AmpC β-lactamases are capable of inactivating broad-spectrum penicillins and cephalosporins, their presence in *Enterobacteriaceae* is of clinical and epidemiological importance (Kolar *et al.*, 2010). Clinically important ESBL-producing *Enterobacteriaceae* have been reported in different South African (SA) provinces (Eastern Cape [Vasaikar *et al.*, 2017]; Western Cape [Peirano *et al.*, 2011]; KwaZulu-Natal [Mahomed and Coovadia, 2014]; and Gauteng Province [Ehlers *et al.*, 2009]). In 53 clinical isolates from Gauteng, ESBL gene prevalence was reported in 87% (Ehlers *et al.*, 2009).

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ORIGINAL RESEARCH
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Occurrence, Phenotypic and Molecular Characterization of Extended-Spectrum- and AmpC-β-Lactamase Producing Enterobacteriaceae Isolated From Selected Commercial Spinach Supply Chains in South Africa

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The increasing occurrence of multidrug-resistant (MDR) extended-spectrum β-lactamase- (ESBL) and/or AmpC β-lactamase-producing Enterobacteriaceae in health care systems, the environment and fresh produce is a serious concern globally. Production practices, processing and subsequent consumption of contaminated raw fruit and vegetables represent a possible human transmission route. The purpose of this study was to determine the presence of ESBL/AmpC-producing Enterobacteriaceae in complete spinach supply chains and to characterize the isolated strains phenotypically (antimicrobial resistance profiles) and genotypically (ESBL/AmpC genetic determinants, detection of class 1, 2, and 3 integrons). Water, soil, fresh produce, and contact surface samples (n = 288) from two commercial spinach production systems were screened for ESBL/AmpC-producing Enterobacteriaceae. In total, 14.58% (42/288) of the samples were found to be contaminated after selective enrichment, plating onto chromogenic media and matrix-assisted laser desorption ionization time-of-flight mass spectrometry identity confirmation of presumptive ESBL/AmpC isolates. This included 15.28% (11/72) water and 12.12% (16/132) harvested- and processed spinach, while 25% (15/60) retail spinach samples were found to be contaminated with an increase in isolate abundance and diversity in both scenarios. Dominant species identified included *Serratia fonticola* (45.86%), *Escherichia coli* (20.83%), and *Klebsiella pneumoniae* (18.75%). In total, 48 (81.36%) isolates were phenotypically confirmed as ESBL/AmpC-producing Enterobacteriaceae of which 98% showed a MDR phenotype. Genotypic characterization (PCR of ESBL/AmpC resistance genes and integrons) further revealed the domination of the CTX-M Group 1 ESBL type, followed by TEM and SHV; whilst the CIT-type was the only plasmid-mediated AmpC genetic determinant detected. Integrons were detected in 79.17% (n = 38) of the confirmed

High prevalence of multidrug resistant *Escherichia coli* isolated from fresh vegetables sold by selected formal and informal traders in the most densely populated Province of South Africa

Loandi Richter, Erika Du Plessis, Stacey Duvenage, and Lise Korsten

Abstract: Contaminated fresh produce has increasingly been implicated in foodborne disease outbreaks. As microbiological safety surveillance in South Africa is limited, a total of 545 vegetable samples (spinach, tomato, lettuce, cucumber, and green beans) were purchased from retailers, street traders, trolley vendors and farmers' markets. *Escherichia coli*, coliforms and Enterobacteriaceae were enumerated and the prevalence of *Escherichia coli*, *Salmonella* spp. and *Listeria monocytogenes* determined. *E. coli* isolates were characterized phenotypically (antibiotic resistance) and genotypically (diarrheagenic virulence genes). Coliforms, *E. coli* and Enterobacteriaceae counts were mostly not significantly different between formal and informal markets, with exceptions noted on occasion. When compared to international standards, 90% to 98% tomatoes, 70% to 94% spinach, 82% cucumbers, 93% lettuce, and 80% green bean samples, had satisfactory (≤ 100 CFU/g) *E. coli* counts. Of the 545 vegetable samples analyzed, 14.86% ($n = 81$) harbored *E. coli*, predominantly from leafy green vegetables. Virulence genes (*lt*, *st*, *bfpA*, *eagg*, *eaeA*, *stx1*, *stx2*, and *ipaH)* were not detected in the *E. coli* isolates ($n = 67$) characterized, however 40.30% were multidrug-resistant. Resistance to aminoglycosides (neomycin, 73.13%; gentamycin, < 10%), penicillins (ampicillin, 38.81%; amoxicillin, 41.79%; augmentin, < 10%), sulfonamides (cotrimoxazole, 22.39%), tetracycline (19.4%), chloramphenicol (11.94%), cephalosporins (cefepime, 34.33%), and carbapenemases (imipenem, < 10%) were observed. This study highlights the need for continued surveillance of multidrug resistant foodborne pathogens in fresh produce retailed formally and informally for potential consumer health risks.

Keywords: Food safety

Practical Application: The results indicate that the microbiological quality of different vegetables were similar per product type, regardless of being purchased from formal retailers or informal street traders, trolley vendors or farmers' markets. Although no pathogenic bacteria (diarrheagenic *E. coli*, *Salmonella* spp. or *L. monocytogenes*) were isolated, high levels of multidrug-resistance was observed in the generic *E. coli* isolates. These findings highlight the importance of microbiological quality surveillance of fresh produce in formal and informal markets, as these products can be a reservoir of multidrug resistant bacteria harboring antibiotic resistance and virulence genes, potentially impacting human health.

1. INTRODUCTION

Surveillance of the microbiological quality of fresh produce at retail level have been reported in various countries (de Oliveira, de Souza, Bergamini, & De Martinis, 2011; Kuan et al., 2017; Li et al., 2017; Roth, Simonne, House, & Ahn, 2018; Ryu, Kim, Kim, Beuchat, & Kim, 2014; Sair, Masud, Ayyaz, & Rafique, 2017; Tango et al., 2018), with increasing numbers being associated with fresh produce resulting in foodborne disease outbreaks (Denis, Zhang, Leroux, Trudel, & Bietlot, 2016). This highlights the need for effective foodborne disease outbreak surveillance and reporting systems in fresh produce supply chains. The South African food market is characterized by dualism; both well-developed, highly sophisticated and regulated formal—as well as the less regulated informal food systems that provide fresh produce to consumers throughout the country (Louw, Chikazunga, Jordaan, & Biénabe, 2006; Skinner & Haysom, 2016). Differences in the production and distribution systems raise the question of possible differences in microbiological quality of the retailed fresh produce (Verraes et al., 2015).

Enterobacteriaceae form part of the indigenous microbiota of vegetables (Blaak, van Hoek, Veenman, Docters van Leeuwen, & Lynch, 2014). Members of this family, that is, *Escherichia coli* and *Salmonella* spp., have often been associated with foodborne bacterial outbreaks following raw fresh produce consumption (Tope, Hitter, & Patel, 2016). This includes diarrheagenic *E. coli* strains, including enteropathogenic (EPEC), enterotoxigenic (ETEC), enterohaemorrhagic (EHEC), enteroaggregative (EAEC), and enteroinvasive (EIEC) *E. coli* in foodborne disease outbreaks (Aijuka, Santiago, Girón, Nataro, & Buys, 2018; Canizalez-Roman et al., 2019). In addition to generic *E. coli*, diarrheagenic strains are also found in the intestinal tracts of mammals and are therefore often used as indicators of fecal contamination in fresh produce supply chains (Denis et al., 2016). Similarly, *Listeria monocytogenes* is

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ORIGINAL ARTICLE

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Microbiological safety of spinach throughout commercial supply chains in Gauteng Province, South Africa and characterization of isolated multidrug-resistant *Escherichia coli*

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Abstract

Aim: To investigate the microbiological quality, potential foodborne pathogen presence, and to phenotypically (antimicrobial resistance [AMR] profiles) and genotypically (DNA fingerprints and diarrhoeagenic genes) characterize *Escherichia coli* isolated throughout spinach production systems from farm-to-sale.

Methods and Results: Samples ($n = 288$) were collected from two commercial supply chains using either river or borehole irrigation water. *E. coli* was enumerated throughout the chain where river water was directly used for overhead irrigation at levels between 0.00 and 3.22 log colony forming unit (CFU) g^{-1}. Following enrichment, isolation and matrix-assisted laser desorption ionization time-of-flight mass spectrometry identification, *E. coli* was isolated from 22.57% ($n = 65/288$) of all samples. *Salmonella* spp. were isolated from 3% ($n = 9/288$) of river and irrigation water samples on one farm, and no *Listeria monocytogenes* was detected throughout the study. Of the 80 characterized *E. coli* isolates, one harboured the *stx2* virulence gene, while 43.75% ($n = 35$) were multidrug resistant. Overall, 26.30% of the multidrug-resistant *E. coli* isolates were from production scenario one that used river irrigation water, and 17.50% from the second production scenario that used borehole irrigation water. A greater percentage of resistance phenotypes were from water *E. coli* isolates (52.50%), than isolates from spinach (37.50%). *E. coli* isolates from spinach and irrigation water clustered together at high similarity values (>90%) using enterobacterial repetitive intergenic consensus-polymerase chan reaction analysis.

Conclusions: This study reported the presence of multidrug-resistant environmental *E. coli* throughout spinach production from farm, during processing and up to retail. Furthermore, the similarity of multi-drug resistant *E. coli* isolates suggests transfer from irrigation water to spinach in both scenarios, reiterating that irrigation water for vegetables consumed raw, should comply with standardized microbiological safety guidelines.

Significance and Impact of Study: Multidrug-resistant *E. coli* presence throughout spinach production emphasizes the necessity of increased surveillance of AMR in fresh produce and the production environment within a One Health paradigm to develop AMR mitigation strategies.

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Whole Genome Sequencing of Extended-Spectrum- and AmpC- β-Lactamase-Positive Enterobacterales Isolated From Spinach Production in Gauteng Province, South Africa

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The increasing occurrence of multidrug-resistant (MDR) extended-spectrum β-lactamase- (ESBL) and/or AmpC β-lactamase- (AmpC) producing Enterobacterales in irrigation water and associated irrigated fresh produce represents risks related to the environment, food safety, and public health. In South Africa, information about the presence of ESBL/AmpC-producing Enterobacterales from non-clinical sources is limited, particularly in the water–plant-food interface. This study aimed to characterize 19 selected MDR ESBL/AmpC-producing *Escherichia coli* ($n = 3$), *Klebsiella pneumoniae* ($n = 5$), *Serratia fonticola* ($n = 10$), and *Salmonella enterica* ($n = 1$) isolates from spinach and associated irrigation water samples from two commercial spinach production systems within South Africa, using whole genome sequencing (WGS). Antibiotic resistance genes potentially encoding resistance to eight different classes were present, with $bla_{CTX\text{-}M\text{-}15}$ being the dominant ESBL encoding gene and bla_{ACT}-types being the dominant AmpC encoding gene detected. A greater number of resistance genes across more antibiotic classes were seen in all the *K. pneumoniae* strains, compared to the other genera tested. From one farm, $bla_{CTX\text{-}M\text{-}15}$-positive *K. pneumoniae* strains of the same sequence type 985 (ST 985) were present in spinach at harvest and retail samples after processing, suggesting successful persistence of these MDR strains. In addition, ESBL-producing *K. pneumoniae* ST15, an emerging high-risk clone causing nosocomical outbreaks worldwide, was isolated from irrigation water. Known resistance plasmid replicon types of Enterobacterales including IncFIB, IncFIA, IncFII, IncB/O, and IncHI1B were observed in all strains following analysis with PlasmidFinder. However, $bla_{CTX\text{-}M\text{-}15}$ was the only β-lactamase resistance gene associated with plasmids (IncFII and IncFIB) in *K. pneumoniae* ($n = 4$) strains. In one *E. coli* and five *K. pneumoniae* strains, integron In191 was observed. Relevant similarities to human pathogens were predicted with PathogenFinder for all 19 strains, with a confidence of

Appendix A

Table A1: The microbiological quality of whole and fresh-cut RTE vegetables that have been analysed for hygiene indicator bacteria and potential foodborne pathogens (*Escherichia coli*, *Salmonella* spp. and/or *Listeria monocytogenes*) in different parts of the world at harvest or at a specific point of sale dating back to 2006

Country where study was conducted	Year	Vegetable type	Whole/RTE bagged and cut	Sampling site	Microbiological quality analysis (Maximum counts, log CFU/g)				Detection of foodborne pathogens (1=detected; 0=not detected)			Reference
					Total aerobic bacteria counts	Coliform counts	*E. coli* counts	Enterobacteriaceae counts	*E. coli*	*Salmonella* spp.	*Listeria* spp.	
Spain	2006	Carrot	Fresh-cut	Retailers	7,8	-	-	5,3	0	0	0	Abadias et al., 2008
		Lettuce	Fresh-cut		6,3	-	-	4,4	1	1	1	
		Spinach	Fresh-cut		7,4	-	-	6	1	1	0	
		Mixed salads	Fresh-cut		7,1	-	-	5,5	1	1	1	
		Iceberg lettuce	Whole		4,6	-	-	2	0	0	0	
		Lettuce hearts	Whole		4,4	-	-	2,5	0	0	0	
		Oakleaf lettuce	Whole		6,7	-	-	3,9	0	0	0	
		Romaine lettuce	Whole		6	-	-	3,7	0	0	0	
Spain	2009	Lettuce	Whole	At harvest	6,35	-	-	5,16	1	0	0	Oliveira et al., 2010
USA	2010	Basil	Whole	Retailers	7,49	4,03	2,08	-	0	0	0	Korir et al., 2016
		Lettuce	Whole		7,76	3	1,3	-	0	0	0	
		Spinach	Whole		8,02	4,53	1,78	-	1	0	1	
		Parsley	Whole		8,02	4,88	1,85	-	0	1	0	
Brazil	2010	Kale	RTE bagged and cut	Retailers	7,8	Analysed as MPN/g	Analysed as MPN/g	-	1	0	1	de Oliveira et al., 2011
		Cabbage	RTE bagged and cut		8,2			-	0	0	0	
		Lettuce	RTE bagged and cut		7,1			-	1	0	0	
		Spring onion and Parsley mix	RTE bagged and cut		9,3			-	1	0	1	
		Chinese cabbage	RTE bagged and cut		7,9			-	0	0	1	

		Spinach	RTE bagged and cut		9			-	0	0	1	
		Watercress	RTE bagged and cut		7,1			-	0	0	0	
Iran	2011	Mixed green leaf vegetables	RTE, fresh-cut	Retailers	8,3	7	≥2	7	1	1	1	Najafi and Bahreini, 2012
		Mixed fresh-cut salads	RTE, fresh-cut		8,3	7,48	≥2	8,3	1	1	0	
Brazil	2011	Loosleaf lettuce	Whole, organic	Farmers' market	7,14	4,32	1,93	-	1	0	-	Maffei et al. 2013
		Butterhead lettuce	Whole, organic		6,73	3,37	1,59	-	1	0	-	
		Romaine lettuce	Whole, organic		6,81	3,5	1,48	-	1	0	-	
		Red looseleaf lettuce	Whole, organic		6,69	3,18	1,16	-	1	0	-	
		Looseleaf lettuce	Whole, conventional		6,5	4,69	1,38	-	1	0	-	
		Butterhead lettuce	Whole, conventional		6,07	3,11	1,3	-	1	0	-	
		Romaine lettuce	Whole, conventional		6,5	3,23	1,58	-	1	0	-	
		Red looseleaf lettuce	Whole, conventional		6,55	4,04	1,23	-	1	0	-	
Saudi Arabia	2012	Lettuce	Whole	Retailers	7,9	5,9	-	5,8	0	0	-	Al-Holy et al., 2013
		Green onion	Whole		8,5	6,2	-	6,8	0	0	-	
		Parsley	Whole		8	6,2	-	6,8	0	0	-	
		Rocket	Whole		8,5	6	-	6	0	0	-	
British Columbia	2012	Green leaf lettuce	Whole	Farmers' market	6,11	2,2	0	-	1	-	-	Wood et al., 2015
		Red leaf lettuce	Whole		6,29	1,6	0	-	1	-	-	
		Romaine lettuce	Whole		6,7	1,9	0	-	1	-	-	
Belgium	2012	Lettuce	Whole	At harvest	6,3	N/A	0,7	-	0	0	N/A	Holvoet et al., 2015
South Africa	2012	Onions	Whole	At harvest	1,6	<0,5	-	-	1	0	0	Du Plessis et al. 2015
		Onions	Whole	Market	0,6	1,7	-	-	1	0	0	
Canada	2013	Leafy vegetables	Whole/RTE bagged and cut	Retailers	-	-	-	-	1	1	1	Denis et al., 2016
		Leafy herbs	Whole		-	-	-	-	1	1	-	
		Tomato	Whole		-	-	-	-	0	0	-	

		Green pepper	Whole		-	-	-	-	1	1	-	
Mexico	2013	RTE Salads	RTE	Supermarkets	4,9	5,6	-	-	1	1		Cerna-Cortes et al., 2015
		RTE Salads	RTE	Street vendor stalls	6,1	1,1	-	-	-	-	-	
Turkey	2013	Green leaf lettuce	Whole	Supermarkets	3,75	3,45	2,5	-	0	1	0	Buyukunal et al., 2015
		Iceberg lettuce	Whole		3,65	3,45	2,25	-	0	1	0	
		Cos lettuce	Whole		3,6	3,4	2,35	-	0	1	0	
		Spinach	Whole		3,6	3,2	0	-	0	1	0	
		Cucumber	Whole		3,35	2,8	0	-	0	0	0	
		Tomato	Whole		3,45	2,95	0	-	0	0	0	
		Green bean	Whole		3,2	2,7	0	-	0	0	0	
		Pepper	Whole		3,2	2,7	0	-	0	0	0	
		Carrot	Whole		3,75	3,25	0	-	0	1	0	
Phillipines	2013	Bell pepper	Whole	Open air market	-	-	3,95	-	1	1		Vital et al., 2014
		Cabbage	Whole		-	-	2,58	-	1	1		
		Carrot	Whole		-	-	4,03	-	1	1		
		Lettuce	Whole		-	-	3,92	-	1	1		
		Tomato	Whole		-	-	3,66	-	1	1		
		Bell pepper	Whole	Retailers	-	-	4,15	-	1	1		
		Cabbage	Whole		-	-	2,88	-	1	1		
		Carrot	Whole		-	-	2,79	-	1	1		
		Lettuce	Whole		-	-	3,15	-	1	1		
		Tomato	Whole		-	-	3,12	-	0	1		
Malawi	2013	Lettuce	Whole	Market	-	4	-	5,3	-	-	-	Mngoli and Austen, 2014
South Africa	2014	Tomato	Whole	At harvest	-	3,2	<1	-	0	0	-	van Dyk et al., 2016
		Tomato	Whole	Informal market	-	4	<1	-	0	0	-	
		Tomato	Whole	Retailers	-	4,7	<1	-	0	0	-	
Oman	2014	Lettuce	Whole	Local markets	±5	-	±1	±4	1	0	0	Al-kharousi et al., 2016
		Cucumber	Whole		±5	-	0	±2	0	0	0	
		Carrot	Whole		±5	-	0	±4	0	0	0	

		Cabbage	Whole		±5	-	±4	±6	1	0	0	
		Tomato	Whole		±5	-	0	±4	0	0	0	
Italy	2014	Spinach	RTE, fresh-cut	Supermarkets	6,95	-	<1	4,9	0	0	0	Cardamone et al., 2015
		Green salad	RTE, fresh-cut		6,98	-	3,95	5,73	0	1	0	
Czech Republic	2014	Mixed vegetables	Fresh-cut	Supermarket	-	-	-	-	-	1	0	Vojkovska et al. 2016
		Spinach	Fresh-cut		-	-	-	-	-	0	1	
		Leafy greens	Fresh-cut		-	-	-	-	-	0	0	
		Rucuola	Fresh-cut		-	-	-	-	-	0	1	
		Cucumber	Whole		-	-	-	-	-	0	0	
		Dill	Whole		-	-	-	-	-	0	0	
		Leafy greens	Whole		-	-	-	-	-	0	0	
		Radish	Whole		-	-	-	-	-	0	0	
		Spring onion	Whole		-	-	-	-	-	0	0	
		Tomato	Whole		-	-	-	-	-	0	0	
South Africa	2015	Cabbage	Whole	Informal street vendors	-	4,03	0,00	-	1	0	0	du Plessis et al., 2017
		Cabbage	Whole	Retailers	-	3,34	0,00	-	1	0	0	
		Spinach	Bunch	Informal street vendors	-	4,97	0,79	-	1	0	0	
		Spinach	Bunch	Retailers	-	4,64	0,37	-	1	0	0	
Germany	2015	Leafy salads	RTE	Retail markets	-	-	3	8,8	0	1	1	Becker et al., 2019
USA	2016	Tomato	Whole	Farmers' market	3,7	3,8	Analysed as MPN/g	-	-	1	1	Li et al., 2017
		Green pepper	Whole		4,5	4		-	-	0	1	
		Cucumber	Whole		4,2	3,7		-	-	0	1	
		Spinach	Whole		7,8	5,4		-	-	1	0	
Rwanda	2016	Beetroot	Whole	At harvest	-	-	-	5,9	-	-	1	Ssemanda et al., 2017
		Cabbage	Whole		-	-	-	5,9	-	-	1	
		Carrot	Whole		-	-	-	5,9	-	-	1	
		Celery	Whole		-	-	-	5,9	-	-	1	
		Cucumber	Whole		-	-	-	5	-	-	1	

		Garlic	Whole		-	-	-	7	-	-	1	
		Green pepper	Whole		-	-	-	4,8	-	-	1	
		Lettuce	Whole		-	-	-	5,3	-	-	1	
		Onion	Whole		-	-	-	7	-	-	1	
		Parsley	Whole		-	-	-	6,2	-	-	1	
		Tomato	Whole		-	-	-	5	-	-	1	
		Beetroot	Whole		-	-	-	6	-	-	1	
		Cabbage	Whole		-	-	-	7	-	-	1	
		Carrot	Whole		-	-	-	7,1	-	-	1	
		Celery	Whole		-	-	-	7,5	-	-	1	
		Cucumber	Whole		-	-	-	5,8	-	-	1	
		Garlic	Whole	Retailers	-	-	-	6,2	-	-	1	
		Green pepper	Whole		-	-	-	5,3	-	-	1	
		Letuce	Whole		-	-	-	6,4	-	-	1	
		Onion	Whole		-	-	-	6,3	-	-	1	
		Parsley	Whole		-	-	-	6,4	-	-	1	
		Tomato	Whole		-	-	-	6	-	-	1	
Malaysia	2016	Cabbage	Whole	Retailers	6,97	4,83	-	-	0	0	1	Kuan et al., 2017
		Carrot	Whole		5,57	4,22	-	-	0	1	1	
		Cherry tomatoes	Whole		3,9	2,09	-	-	0	0	0	
		Cucumber	Whole		5,55	2,48	-	-	0	1	1	
		Lettuce	Whole		6,7	4,61	-	-	1	0	1	
		Tomato	Whole		4,9	1	-	-	0	0	0	
Pakistan	2016	Tomato	Whole	Retailers	7	5,8	-	-	1	-	-	Sair et al., 2017
		Carrot	Whole		6,6	5,1	-	-	1	-	-	
		Green pepper	Whole		5,8	3,7	-	-	1	-	-	
		Cucumber	Whole		5,1	3,8	-	-	1	-	-	
		Onion	Whole		4,1	2,7	-	-	1	-	-	
		Lettuce	Whole		7	6,2	-	-	1	-	-	
		Cabbage	Whole		7,3	6,1	-	-	1	-	-	

		Mixed fresh-cut salads	RTE, fresh-cut		9	8	-	-	1	-	-	
China	2016	Coriander	Not Specified	Retailers	-	-	-	-	-	1	-	Olivera et al., 2019
		Lettuce	Not Specified		-	-	-	-	-	1	-	
		Tomato	Not Specified		-	-	-	-	-	1	-	
		Cucumber	Not Specified		-	-	-	-	-	1	-	
Phillipines	2016	Bell pepper	Not Specified	Open air market	-	-	-	-	1	1	-	Vital et al., 2019
		Carrot	Not Specified	Open air market	-	-	-	-	1	1	-	
		Lettuce	Not Specified	Open air market	-	-	-	-	1	1	-	
		Tomato	Not Specified	Open air market	-	-	-	-	1	1	-	
		Bell pepper	Not Specified	Supermarkets	-	-	-	-	0		-	
		Carrot	Not Specified	Supermarkets	-	-	-	-	0	0	-	
		Lettuce	Not Specified	Supermarkets	-	-	-	-	1	0	-	
		Tomato	Not Specified	Supermarkets	-	-	-	-	0	0	-	
USA	2017	Leafy greens	Whole and pre-bagged	Farmers' market	-	2,3	1,88	-	0	0	1	Roth et al., 2018
		Spinach	Bunch, pre-cut		-	2,4	2,01	-	0	0	1	
		Tomato	Whole		-	1,6	<1	-	0	1	0	
		Leafy greens	Whole and pre-bagged	Supermarkets	-	1,1	-	-	0	0	0	
		Spinach	Bunch, pre-cut		-	0,7	-	-	0	0	0	
		Tomato	Whole		-	1,4	-	-	0	0	0	
Korea	2017	Chinese cabbage	Whole	At harvest	-	4,74	0	-	0	0	0	Song et al., 2019
		Romaine lettuce	Whole		-	5,65	0	-	0	0	0	
		Cucumber	Whole		-	4,18	2	-	0	0	0	
		Pepper	Whole		-	1,63	0	-	0	0	0	
		Tomato	Whole		-	2,97	0	-	0	0	0	
India	2018	Beetroot	Whole	At harvest	-	2,12	0,86	-	1	-	-	Pushpakanth et al., 2019
		Cabbage	Whole		-	2,86	1,96	-	1	-	-	
		Carrot	Whole		-	2,9	2,06	-	1	-	-	
		Parsley	Whole		-	0,44	0	-	0	-	-	

		Potato	Whole			1,96	1,08	-	1	-	-	
Italy	2018	RTE Mixed Salad	Raw material	Industry	6,7	-	-	-	0	0	0	Calonico et al., 2019
		RTE Mixed Salad	Mixed leaves after 2nd washing		5,9	-	-	-	0	0	0	
		RTE Mixed Salad	Mixed leaves after 5th washing		5,7	-	-	-	0	0	0	
		RTE Mixed Salad	RTE packaged		5,2	-	-	-	0	0	0	
		RTE Mixed Salad	RTE- end of shelf life		7,9	-	-	-	0	0	0	
		RTE Mixed Salad	RTE packaged	Supermarkets	7,1	-	2,5	-	0	0	0	
		RTE Mixed Salad	RTE packaged and washed		7	-	0	-	0	0	0	
		RTE Mixed Salad	RTE packaged end of shelf life		7,3	-	2,5	-	0	0	0	

Table A2: Subgroups of fruits and vegetables based on food component content and classification variables (botanic family, plant part, colour, and total antioxidant capacity)

Subgroup	Name	Nr of Fruit	Nr of Vegetables	Fruits and Vegetables in subgroup
1	Dark green leafy vegetables	0	9	Spinach, beet greens, kale, collards, parsley, mustard greens, Swiss chard, turnip greens, romaine
2	Cabbage family vegetables	0	8	Chinese cabbage, broccoli, Brussels sprouts, broccoli raab, cabbage (green and red), Chinese broccoli, cauliflower
3	Lettuces	0	6	Watercress, butterhead lettuce, iceberg lettuce, endive, leaf lettuce (green and red)
4	Legumes	0	10	Green peas, lentils, lima beans (immature), blackeye peas (mature), kidney beans (mature), navy beans (mature), mung beans (mature), pigeion peas (mature), soybeans (mature), pinto beans (mature)
5	Allium family bulbs	0	4	Onion, garlic, leek, scallion
6	Deep orange/yellow gruits, roots, and tubers	6	5	Cantaloupe, apricot, mango, nectarine, papaya, peach, butternut squash, carrot, pumpkin, hubbard squash, sweet potato
7	Tomatoes and other red vegetables and fruits	4	4	Watermelon, cherries, guava, pomegranate, beet, rhubarb, tomato, red pepper
8	Citrus family fruits	8	0	Grapefruit (white and pink), clementine, kumquat, lime, lemon, orange, tangerine
9	Red/purple/blue berries	6	0	Cranberries, blackberries, blueberries, raspberries, boysenberries, strawberries
10	Other	14	20	Apple, Asian pear, artichoke, banana, casaba melon, fig, date, grapes, kiwi, honedew melon, pineapple, pear, plum, raisins, asparagus, celery, avocado, corn, cucumber, eggplant, green pepper, crookneck squash, Jerusalem artichoke, okra, jicama, okra parsnip, potato, radish, rutabaga snap beans, snowpeas, turnip, zucchini

Appendix B

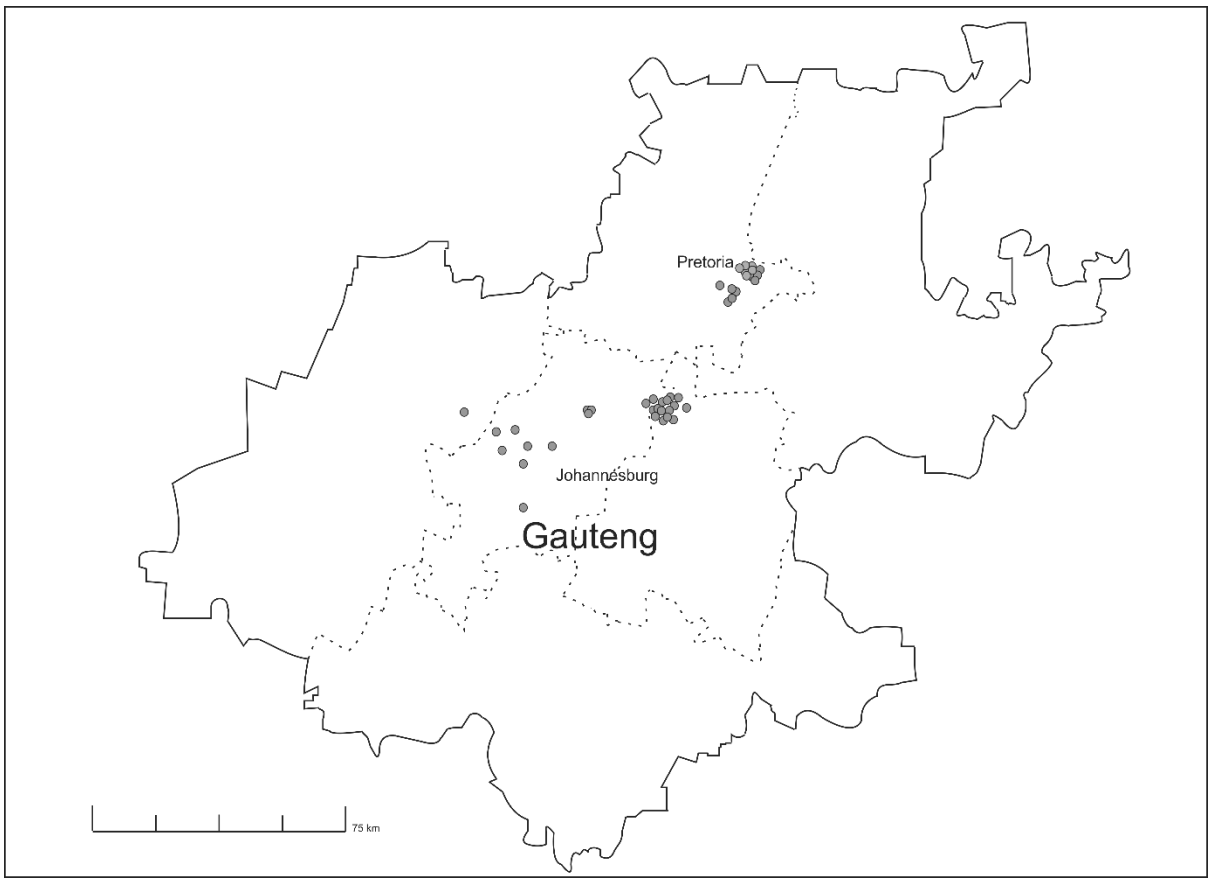

Figure B1: Map of Gauteng, Province South Africa, showing the sampling sites where vegetables were purchased at formal and informal markets.

Appendix B

Table B2: Total coliform, *Escherichia coli* and Enterobacteriaceae loads present in spinach, lettuce, cucumber and green bean samples purchased from retailers, street trading greengrocers, trolley vendors, and vendors at farmers' markets in Gauteng Province, South Africa.

		Total coliforms (log CFU/g)			***E. coli* (log CFU/g)**			**Enterobacteriaceae (log CFU/g)**	
Product	No of samples (% harbouring coliforms)	Range	Mean[a]	No of samples (% harbouring *E. coli*)	Range	Mean[a]	No of samples (% harbouring Enterobacteriaceae)	Range	Mean[a]
Spinach									
Retailers	50 (100)	2.90 - 7.17	5.61^{AB}	50 (20)	0.00 - 3.42	0.84^{AB}	50 (100)	2.78 - 8.16	5.79^{ABC}
Street traders	50 (100)	0.70 - 7.60	5.54^{AB}	50 (12)	0.00 - 2.08	0.25^{BC}	50 (98)	0.00 - 6.99	5.42^{ABCD}
Trolley vendors	50 (100)	0.59 - 7.04	5.05^{BCD}	50 (28)	0.00 - 1.29	0.72^{ABC}	50 (90)	0.00 - 7.27	6.63^{DE}
Farmers' market vendors	50 (100)	3.76 - 8.10	6^{A}	50 (44)	0.00 - 5.88	1.22^{A}	50 (100)	4.03 - 7.88	5.92^{AB}
Total for spinach	**200**								
Tomato									
Retailers	50 (100)	0.48 - 8.04	4.58^{CDE}	50 (94)	0.00 - 0.89	0.12^{C}	50 (100)	2.40 - 8.10	5.34^{ABCD}
Street traders	50 (100)	2.00 - 8.21	4.96^{BCDE}	50 (100)	0.00 - 2.30	0.05^{C}	50 (98)	0.00 - 7.82	4.76^{CDE}
Trolley vendors	50 (100)	0.00 - 6.36	4.42^{DE}	50 (98)	0.00 - 3.60	0.16^{BC}	50 (92)	0.00 - 7.94	4.51^{DE}
Farmers' market vendors	50 (100)	3.15 - 7.89	5.43^{ABC}	50 (20)	0.00 - 5.10	0.54^{ABC}	50 (100)	1.49 - 7.75	5.02^{BCDE}
Total for tomato	**200**								
Lettuce									
Farmers' market vendors	50 (100)	3.58 - 7.82	6.08^{A}	50 (26)	0.00 - 3.31	0.65^{ABC}	50 (100)	4.18 - 8.26	6.22^{A}
Total for lettuce	**50**								
Cucumber									
Farmers' market vendors	45 (96)	0.00 - 6.48	4.06^{E}	45 (20)	0.00 - 3.78	0.43^{BC}	45 (96)	0.00 - 6.45	4^{E}
Total for cucumber	**45**								
Green beans									
Farmers' market vendors	50 (100)	0.70 - 6.77	4.97^{BCDE}	50 (28)	0.00 - 4.78	0.68^{ABC}	50 (98)	0.00 - 6.71	5.22^{ABCD}
Total for green beans	**50**								

[a]Within each column, means (based on the product interactions) followed by the same letters are not significantly different (p <0.05).

Table B3: Summary of the number of antimicrobials, most frequent resistance patterns, number, and type of antibiotic classes to which generic *Escherichia coli* isolates from different fresh produce samples were resistant

No of antimicrobials to which isolates were resistant	No of isolates (n=67)	No of isolates with specific pattern	Most frequent pattern[a]	No of antibiotic classes to which isolates were resistant	Antibiotic class(es)
0	8				
1	21	17	NE10C	1	Aminoglycosides
		2	T30C	1	Tetraclyclines
		1	AUG30C	1	Penicillins
		1	CPM30C	1	Cephalosporins
2	8	2	AUG30C - NE10C	2	Penicillins, Aminoglycosides
		1	AP10C - NE10C	2	Penicillins, Aminoglycosides
		1	TS25C - T30C	2	Sulfonamides, Tetracyclines
		1	A10C - NE10C	2	Penicillins, Aminoglycosides
		1	A10C - CPM30C	2	Penicillins, Cephalosporins
		1	CPM30C - NE10C	2	Cephalosporins, Aminoglycosides
		1	GM10C - NE10C	1	Aminoglycosides
3	5	3	A10C - CPM30C - NE10C	3	Penicillins, Cephalosporins, Aminoglycosides
		1	AP10C - A10C - CPM30C	2	Penicillins, Cephalosporins
		1	AP10C - A10C - NE10C	2	Penicillins, Aminoglycosides
4	12	7	AP10C - A10C - CPM30C - NE10C	3	Penicillins, Cephalosporins, Aminoglycosides
		1	FOX30C - CPM30C - TS25C - NE10C	3	Cephalosporins, Sulfonamides, Aminoglycosides
		1	AP10C - AUG30C - A10C - NE10C	2	Penicillins, Aminoglycosides
		1	AP10C - CPM30C - TS25C - NE10C	4	Penicillins, Cephalosporins, Sulfonamides, Aminoglycosides
		1	AP10C - A10C - CPM30C - TS25C	3	Penicillins, Cephalosporins, Sulfonamides
		1	TS25C - T30C - NE10C - C30C	4	Sulfonamides, Tetracyclines, Aminoglycosides, Chloramphenicol
5	6	1	AP10C - A10C - T30C - NE10C - C30C	4	Penicillins, Tetracyclines, Aminoglycosides, Chloramphenicol
		1	AP10C - A10C - CPM30C - TS25C - NE10C	4	Penicillins, Cephalosporins, Sulfonamides, Aminoglycosides
		1	AP10C - AUG30C - A10C - CPM30C - NE10C	3	Penicillins, Cephalosporins, Aminoglycosides
		1	AP10C - A10C - CPM30C - TS25C - IMI10C	4	Penicillins, Cephalosporins, Sulfonamides, Carbapenems
		1	AP10C - A10C - CPM30C - T30C - NE10C	4	Penicillins, Cephalosporins, Tetracyclines, Aminiglycosides
		1	AP10C - TS25C - T30C - NE10C - C30C	5	Penicillins, Sulfonamides, Tetracyclines, Aminoglycosides, Chloramphenicol
6	6	4	AP10C - A10C - TS25C - T30C - NE10C - C30C	5	Penicillins, Sulfonamides, Tetracyclines, Aminoglycosides, Chloramphenicol
		1	AP10C - A10C - CPM30C - TS25C - T30C - NE10C	5	Penicillins, Cephalosporins, Sulfonamides, Tetracyclines, Aminoglycosides
		1	AP10C - AUG30C - A10C - CPM30C - TS25C - NE10C	4	Penicillins, Cephalosporins, Sulfonamides, Aminoglycosides
7	1	1	AP10C - AUG30C - A10C - TS25C - T30C - NE10C - C30C	5	Penicillins, Sulfonamides, Tetracyclines, Aminoglycosides, Chloramphenicol

Appendix B

Table B4: Matrix-assisted laser desorption time-of-flight identification of *Escherichia coli* isolated from fresh produce sold formally and informally in Gauteng Province

University of Pretoria Culture number	UPMP code	Published isolate number	Sample Code	Farm/Vendor	Source	Organism Identity	Organism best match*		Range**	Consistency category***	Notes	Isolated by
							Score 1	Score 2				
UP_BN_LR_0326	UPMP 891	1	2C5.2 16	Farmers' market	Cucumber	*Escherichia coli*	2.485	2.413	+++	A	Generic	Loandi Richter
UP_BN_LR_0324	UPMP 889	2	2C4.2 14	Farmers' market	Cucumber	*Escherichia coli*	2.42	2.351	+++	A	Generic	Loandi Richter
UP_BN_LR_0347	UPMP 912	3	F3e	Farmers' market_Cucumber	Cucumber	*Escherichia coli*	2.451	2.449	+++	A	Generic	Loandi Richter
UP_BN_LR_0348	UPMP 913	4	F4e	Farmers' market_Cucumber	Cucumber	*Escherichia coli*	2.477	2.406	+++	A	Generic	Loandi Richter
UP_BN_LR_0286	UPMP 851	5	C2 1	Farmers' market	Cucumber	*Escherichia coli*	2.552	2.519	+++	A	Generic	Loandi Richter
UP_BN_LR_0287	UPMP 852	6	C4.1 2	Farmers' market	Cucumber	*Escherichia coli*	2.521	2.491	+++	A	Generic	Loandi Richter
UP_BN_LR_0288	UPMP 853	7	C4.2 3	Farmers' market	Cucumber	*Escherichia coli*	2.497	2.439	+++	A	Generic	Loandi Richter
UP_BN_LR_0304	UPMP 869	8	5C1.6 20	Farmers' market	Cucumber	*Escherichia coli*	2.453	2.438	+++	A	Generic	Loandi Richter
UP_BN_LR_0310	UPMP 875	9	5C5 27	Farmers' market	Cucumber	*Escherichia coli*	2.59	2.515	+++	A	Generic	Loandi Richter
UP_BN_LR_0321	UPMP 886	10	2C2.1 11	Farmers' market	Cucumber	*Escherichia coli*	2.418	2.362	+++	A	Generic	Loandi Richter
UP_BN_LR_0349	UPMP 914	11	F6e	Farmers' market_Green beans	Green beans	*Escherichia coli*	2.53	2.421	+++	A	Generic	Loandi Richter
UP_BN_LR_0356	UPMP 921	12	F21e	Farmers' market_Green beans	Green beans	*Escherichia coli*	2.521	2.429	+++	A	Generic	Loandi Richter
UP_BN_LR_0354	UPMP 919	13	F15e	Farmers' market_Green beans	Green beans	*Escherichia coli*	2.511	2.474	+++	A	Generic	Loandi Richter
UP_BN_LR_0352	UPMP 917	14	F12e	Farmers' market_Green beans	Green beans	*Escherichia coli*	2.401	2.394	+++	A	Generic	Loandi Richter
UP_BN_LR_0346	UPMP 911	15	F2e	Farmers' market_Green beans	Green beans	*Escherichia coli*	2.472	2.457	+++	A	Generic	Loandi Richter
UP_BN_LR_0295	UPMP 860	16	5B5.1 2	Farmers' market	Green beans	*Escherichia coli*	2.4	2.369	+++	A	Generic	Loandi Richter
UP_BN_LR_0297	UPMP 862	17	1B3.1 6	Farmers' market	Green beans	*Escherichia coli*	2.384	2.34	+++	A	Generic	Loandi Richter
UP_BN_LR_0298	UPMP 863	18	1B4 7	Farmers' market	Green beans	*Escherichia coli*	2.355	2.289	+++	A	Generic	Loandi Richter
UP_BN_LR_0314	UPMP 879	19	2B1.3 4	Farmers' market	Green beans	*Escherichia coli*	2.465	2.453	+++	A	Generic	Loandi Richter
UP_BN_LR_0315	UPMP 880	20	2B3 5	Farmers' market	Green beans	*Escherichia coli*	2.427	2.404	+++	A	Generic	Loandi Richter
UP_BN_LR_0320	UPMP 885	21	2B5.3 10	Farmers' market	Green beans	*Escherichia coli*	2.446	2.446	+++	A	Generic	Loandi Richter

UP_BN_LR_0350	UPMP 915	22	F7e	Farmers' market_Lettuce	Lettuce	*Escherichia coli*	2.466	2.424	+++	A	Generic	Loandi Richter
UP_BN_LR_0333	UPMP 898	23	3L4 26	Farmers' market	Lettuce	*Escherichia coli*	2.427	2.406	+++	A	Generic	Loandi Richter
UP_BN_LR_0332	UPMP 897	24	3L3 25	Farmers' market	Lettuce	*Escherichia coli*	2.531	2.437	+++	A	Generic	Loandi Richter
UP_BN_LR_0343	UPMP 908	25	5L2 37	Farmers' market	Lettuce	*Escherichia coli*	2.497	2.496	+++	A	Generic	Loandi Richter
UP_BN_LR_0355	UPMP 920	26	F18e	Farmers' market_Lettuce	Lettuce	*Escherichia coli*	2.503	2.438	+++	A	Generic	Loandi Richter
UP_BN_LR_0386	UPMP 951	27	e149	Farmers' market_Lettuce	Lettuce	*Escherichia coli*	2.448	2.448	+++	A	Generic	Loandi Richter
UP_BN_LR_0364	UPMP 929	28	F60e	Farmers' market_Lettuce	Lettuce	*Escherichia coli*	2.52	2.484	+++	A	Generic	Loandi Richter
UP_BN_LR_0388	UPMP 953	29	e151	Farmers' market_Lettuce	Lettuce	*Escherichia coli*	2.397	2.367	+++	A	Generic	Loandi Richter
UP_BN_LR_0375	UPMP 940	30	e112	Farmers' market_Lettuce	Lettuce	*Escherichia coli*	2.425	2.364	+++	A	Generic	Loandi Richter
UP_BN_LR_0344	UPMP 909	31	5L4 38	Farmers' market	Lettuce	*Escherichia coli*	2.423	2.418	+++	A	Generic	Loandi Richter
UP_BN_LR_0345	UPMP 910	32	5L5 39	Farmers' market	Lettuce	*Escherichia coli*	2.43	2.381	+++	A	Generic	Loandi Richter
UP_BN_LR_0384	UPMP 949	33	e141	Farmers' market_Lettuce	Lettuce	*Escherichia coli*	2.491	2.41	+++	A	Generic	Loandi Richter
UP_BN_LR_0373	UPMP 938	34	e110	Farmers' market_Lettuce	Lettuce	*Escherichia coli*	2.53	2.445	+++	A	Generic	Loandi Richter
UP_BN_LR_0381	UPMP 946	35	e133	Farmers' market_Lettuce	Lettuce	*Escherichia coli*	2.39	2.354	+++	A	Generic	Loandi Richter
UP_BN_LR_0383	UPMP 948	36	e140	Farmers' market_Lettuce	Lettuce	*Escherichia coli*	2.418	2.38	+++	A	Generic	Loandi Richter
UP_BN_LR_0293	UPMP 858	37	L4 12	Farmers' market	Lettuce	*Escherichia coli*	2.503	2.444	+++	A	Generic	Loandi Richter
UP_BN_LR_0300	UPMP 865	38	1L2.4 13	Farmers' market	Lettuce	*Escherichia coli*	2.288	2.275	+++	A	Generic	Loandi Richter
UP_BN_LR_0335	UPMP 900	39	4S2.1 28	Farmers' market	Spinach	*Escherichia coli*	2.569	2.561	+++	A	Generic	Loandi Richter
UP_BN_LR_0336	UPMP 901	40	4S3 30	Farmers' market	Spinach	*Escherichia coli*	2.542	2.447	+++	A	Generic	Loandi Richter
UP_BN_LR_0369	UPMP 934	41	F86e	Farmers' market_Spinach	Spinach	*Escherichia coli*	2.331	2.248	+++	A	Generic	Loandi Richter
UP_BN_LR_0378	UPMP 943	42	e123	Farmers' market_Spinach	Spinach	*Escherichia coli*	2.452	2.403	+++	A	Generic	Loandi Richter
UP_BN_LR_0334	UPMP 899	43	4S1 27	Farmers' market	Spinach	*Escherichia coli*	2.459	2.457	+++	A	Generic	Loandi Richter
UP_BN_LR_0337	UPMP 902	44	4S4 31	Farmers' market	Spinach	*Escherichia coli*	2.494	2.418	+++	A	Generic	Loandi Richter
UP_BN_LR_0360	UPMP 925	45	F41e	Farmers' market_Spinach	Spinach	*Escherichia coli*	2.146	2.136	+++	A	Generic	Loandi Richter
UP_BN_LR_0363	UPMP 928	46	F54e	Farmers' market_Spinach	Spinach	*Escherichia coli*	2.419	2.401	+++	A	Generic	Loandi Richter
UP_BN_LR_0395	UPMP 960	47	R4e (RT2S2)	Retailer2_Spinach	Spinach	*Escherichia coli*	2.418	2.345	+++	A	Generic	Loandi Richter
UP_BN_LR_0394	UPMP 959	48	R1e (RT2S3	Retailer2_Spinach	Spinach	*Escherichia coli*	2.522	2.471	+++	A	Generic	Loandi Richter

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UP_BN_LR_0385	UPMP 950	49	e143	Farmers' market_Spinach	Spinach	*Escherichia coli*	2.42	2.413	+++	A	Generic	Loandi Richter
UP_BN_LR_0368	UPMP 933	50	F79e	Farmers' market_Spinach	Spinach	*Escherichia coli*	2.509	2.495	+++	A	Generic	Loandi Richter
UP_BN_LR_0392	UPMP 957	51	I3e (GG2S5)	Greengrocer2_Spinach	Spinach	*Escherichia coli*	2.467	2.467	+++	A	Generic	Loandi Richter
UP_BN_LR_0391	UPMP 956	52	I2e (GG2S2)	Greengrocer2_Spinach	Spinach	*Escherichia coli*	2.527	2.43	+++	A	Generic	Loandi Richter
UP_BN_LR_0396	UPMP 961	53	R5e (RT2S1)	Retailer2_Spinach	Spinach	*Escherichia coli*	2.528	2.448	+++	A	Generic	Loandi Richter
UP_BN_LR_0390	UPMP 955	54	I1e (GG2S1)	Greengrocer2_Spinach	Spinach	*Escherichia coli*	2.486	2.417	+++	A	Generic	Loandi Richter
UP_BN_LR_0290	UPMP 855	55	S3.2 7	Farmers' market	Spinach	*Escherichia coli*	2.455	2.378	+++	A	Generic	Loandi Richter
UP_BN_LR_0292	UPMP 857	56	S1.3 11	Farmers' market	Spinach	*Escherichia coli*	2.425	2.378	+++	A	Generic	Loandi Richter
UP_BN_LR_0299	UPMP 864	57	3S4 9	Farmers' market	Spinach	*Escherichia coli*	2.492	2.438	+++	A	Generic	Loandi Richter
UP_BN_LR_0311	UPMP 876	58	1S4 1	Farmers' market	Spinach	*Escherichia coli*	2.477	2.47	+++	A	Generic	Loandi Richter
UP_BN_LR_0331	UPMP 896	59	2T5.1 23	Farmers' market	Tomato	*Escherichia coli*	2.466	2.404	+++	A	Generic	Loandi Richter
UP_BN_LR_0330	UPMP 895	60	2T4.2 22	Farmers' market	Tomato	*Escherichia coli*	2.443	2.442	+++	A	Generic	Loandi Richter
UP_BN_LR_0340	UPMP 905	61	4T1.3 34	Farmers' market	Tomato	*Escherichia coli*	2.526	2.519	+++	A	Generic	Loandi Richter
UP_BN_LR_0328	UPMP 893	62	2T2.2 20	Farmers' market	Tomato	*Escherichia coli*	2.399	2.394	+++	A	Generic	Loandi Richter
UP_BN_LR_0357	UPMP 922	63	F28e	Farmers' market_Tomato	Tomato	*Escherichia coli*	2.453	2.448	+++	A	Generic	Loandi Richter
UP_BN_LR_0382	UPMP 947	64	e138	Farmers' market_Tomato	Tomato	*Escherichia coli*	2.431	2.425	+++	A	Generic	Loandi Richter
UP_BN_LR_0342	UPMP 907	65	4T4.2 36	Farmers' market	Tomato	*Escherichia coli*	2.449	2.403	+++	A	Generic	Loandi Richter
UP_BN_LR_0393	UPMP 958	66	I4e (GG2T5)	Greengrocer2_Tomato	Tomato	*Escherichia coli*	2.449	2.403	+++	A	Generic	Loandi Richter
UP_BN_LR_0359	UPMP 924	67	F35e	Farmers' market_Tomato	Tomato	*Escherichia coli*	2.485	2.427	+++	A	Generic	Loandi Richter

Appendix C

Table C1: Matrix-assisted laser desorption time-of-flight identification of extended-spectrum β-lactamase-producing Enterobacteriaceae isolated from fresh produce sold formally and informally in Gauteng Province

University of Pretoria Culture number	UPMP code	Published isolate number	Isolate Code	Farm/Vendor	Source	Presumptive Organism Identity	Organism best match*		Range**	Consistency category***	Notes	Isolated by
							Score 1	Score 2				
UP_BN_LR_0186	UPMP 751	1	E4.1 GG5 S5	Street vendor	Spinach	*Achromobacter xylosoxidans*	2.254	2.158	++	A	ESBL	Loandi Richter
UP_BN_LR_0143	UPMP 708	2	F2 MT2 S4	Mobile trolly	Spinach	*Achromobacter xylosoxidans*	2.276	2.193	++	B	ESBL	Loandi Richter
UP_BN_LR_0264	UPMP 829	3	RT3T2 (R17)	Retailer	Tomato	*Citrobacter farmeri*	2.28	2.246	++	B	ESBL	Loandi Richter
UP_BN_LR_0243	UPMP 808	4	5T1 (F97)	Farmers' market	Tomato	*Citrobacter freundii*	2.367	2.292	+++	B	ESBL	Loandi Richter
UP_BN_LR_0256	UPMP 821	5	5T1 (F110)	Farmers' market	Tomato	*Citrobacter freundii*	2.298	2.173	++	A	ESBL	Loandi Richter
UP_BN_LR_0158	UPMP 723	6	B2.2 GG1 S3.1	Street vendor	Spinach	*Escherichia coli*	2.423	2.374	+++	A	ESBL	Loandi Richter
UP_BN_LR_0159	UPMP 724	7	B2.2 GG2 S3.1	Street vendor	Spinach	*Escherichia coli*	2.534	2.52	+++	A	ESBL	Loandi Richter
UP_BN_LR_0213	UPMP 778	8	C4.1 RT1 S5	Retailer	Spianch	*Escherichia coli*	2,19	2,147	++	C	ESBL	Loandi Richter
UP_BN_LR_0203	UPMP 768	9	C4.2 RT2 S3 2.1	Retailer	Spinach	*Escherichia coli*	2,06	2,206	++	A	ESBL	Loandi Richter
UP_BN_LR_0219	UPMP 784	10	1S2 (3.2)	Farmers' market	Spinach	*Escherichia coli*	2.431	2.428	+++	A	ESBL	Loandi Richter
UP_BN_LR_0220	UPMP 785	11	1S3 (3.3)	Farmers' market	Spinach	*Escherichia coli*	2.494	2.44	+++	A	ESBL	Loandi Richter
UP_BN_LR_0221	UPMP 786	12	1S5 (3.4)	Farmers' market	Spinach	*Escherichia coli*	2.352	2.267	+++	A	ESBL	Loandi Richter
UP_BN_LR_0138	UPMP 703	13	B3 MT1 S2	Mobile trolly	Spinach	*Escherichia coli*	2.417	2.219	+++	B	ESBL	Loandi Richter
UP_BN_LR_0160	UPMP 725	14	B3 GG3 T1	Street vendor	Tomato	*Escherichia coli*	1.957	1.897	+	B	ESBL	Loandi Richter
UP_BN_LR_0201	UPMP 766	15	C4.2 RT2 S3 2.2	Retailer	Spinach	*Escherichia coli*	2.008	1.956	++	A	ESBL	Loandi Richter
UP_BN_LR_0204	UPMP 769	16	C4.2 RT2 S4 1.2	Retailer	Spinach	*Escherichia coli*	2.339	2.224	+++	B	ESBL	Loandi Richter
UP_BN_LR_0224	UPMP 789	17	GG2S1 (I9)	Street vendor	Spinach	*Escherichia coli*	2.449	2.424	+++	A	ESBL	Loandi Richter
UP_BN_LR_0226	UPMP 791	18	GG2T5 (I18)	Street vendor	Tomato	*Escherichia coli*	2.58	2.453	+++	A	ESBL	Loandi Richter
UP_BN_LR_0244	UPMP 809	19	5T3 (F98)	Farmers' market	Tomato	*Eschericia coli*	2.347	2.286	+++	A	ESBL	Loandi Richter
UP_BN_LR_0245	UPMP 810	20	5B5 (F99)	Farmers' market	Green beans	*Escherichia coli*	2.462	2.401	+++	A	ESBL	Loandi Richter

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UP_BN_LR_0246	UPMP 811	21	5S1 (F100)	Farmers' market	Spinach	*Escherichia coli*	2.414	2.377	+++	A	ESBL	Loandi Richter
UP_BN_LR_0248	UPMP 813	22	5S3 (F102)	Farmers' market	Spinach	*Escherichia coli*	2.461	2.447	+++	A	ESBL	Loandi Richter
UP_BN_LR_0249	UPMP 814	23	5S5 (F103)	Farmers' market	Spinach	*Escherichia coli*	2.449	2.431	+++	A	ESBL	Loandi Richter
UP_BN_LR_0250	UPMP 815	24	5S4 (F104)	Farmers' market	Spinach	*Escherichia coli*	2.275	2.193	++	A	ESBL	Loandi Richter
UP_BN_LR_0254	UPMP 819	25	5B5(F108)	Farmers' market	Green beans	*Escherichia coli*	2.469	2.346	+++	A	ESBL	Loandi Richter
UP_BN_LR_0172	UPMP 737	26	A1 LT 1.2 B	Street vendor	Tomato	*Enterobacter asburiae*	2.347	2.286	+++	A	ESBL	Loandi Richter
UP_BN_LR_0255	UPMP 820	26	5T3 (F109)	Farmers' market	Tomato	*Escherichia coli*	2.389	2.296	+++	A	ESBL	Loandi Richter
UP_BN_LR_0136	UPMP 701	27	A4 MTT 4.1 B	Mobile trolly	Tomato	*Enterobacter asburiae*	2,305	2,342	+++	A	ESBL	Loandi Richter
UP_BN_LR_0148	UPMP 713	28	F2 MT3 T1	Mobile trolly	Tomato	*Enterobacter asburiae*	2,382	2,364	+++	A	ESBL	Loandi Richter
UP_BN_LR_0263	UPMP 828	29	RT3S1 (R12)	Retailer	Spinach	*Enterobacter asburiae*	2.276	2.203	++	B	ESBL	Loandi Richter
UP_BN_LR_0259	UPMP 824	30	RT1T2 (R3)	Retailer	Tomato	*Enterobacter asburiae*	2.241	2.177	++	B	ESBL	Loandi Richter
UP_BN_LR_0145	UPMP 710	31	F2 MT2 S1	Mobile trolly	Spinach	*Enterobacter cloacae*	2.45	2.415	+++	A	ESBL	Loandi Richter
UP_BN_LR_0149	UPMP 714	32	F2 MT3 S4	Mobile trolly	Spinach	*Enterobacter cloacae*	2.465	2.346	+++	A	ESBL	Loandi Richter
UP_BN_LR_0173	UPMP 738	33	A1 LT 2.1 B	Street vendor	Tomato	*Enterobacter cloacae*	2.486	2.43	+++	A	ESBL	Loandi Richter
UP_BN_LR_0190	UPMP 755	34	E4 GG5 T5 2.	Street vendor	Tomato	*Enterobacter cloacae*	2.613	2.538	+++	A	ESBL	Loandi Richter
UP_BN_LR_0150	UPMP 715	35	F2 MT3 T3 1.	Mobile trolly	Tomato	*Enterobacter cloacae*	2.544	2.495	+++	A	ESBL	Loandi Richter
UP_BN_LR_0217	UPMP 782	36	1T2 (1.5)	Farmers' market	Tomato	*Enterobacter cloacae*	2.417	2.219	+++	B	ESBL	Loandi Richter
UP_BN_LR_0247	UPMP 812	37	5S2 (F101)	Farmers' market	Spinach	*Enterobacter cloacae*	1.957	1.897	+	B	ESBL	Loandi Richter
UP_BN_LR_0235	UPMP 800	38	4C3 (F66)	Farmers' market	Cucumber	*Enterobacter cloacae*	2.008	1.956	++	A	ESBL	Loandi Richter
UP_BN_LR_0236	UPMP 801	39	3C5 (F68)	Farmers' market	Cucumber	*Enterobacter cloacae*	2.339	2.224	+++	B	ESBL	Loandi Richter
UP_BN_LR_0218	UPMP 783	40	1T3 (1.19)	Farmers' market	Tomato	*Enterobacter cowanii*	1.954	1.791	+	C	ESBL	Loandi Richter
UP_BN_LR_0211	UPMP 776	41	D4.1 RT5 S5	Retailer	Spinach	*Enterobacter ludwigii*	2.062	1.962	++	C	ESBL	Loandi Richter
UP_BN_LR_0156	UPMP 721	42	B2 GG2 S3	Street vendor	Spinach	*Klebsiella pneumoniae*	2.303	2.302	+++	A	ESBL	Loandi Richter
UP_BN_LR_0196	UPMP 761	45	C4.1 RT2 S1	Retailer	Spinach	*Klebsiella pneumoniae*	2.415	2.398	+++	A	ESBL	Loandi Richter
UP_BN_LR_0178	UPMP 743	46	A1 LT 4.1 Ox	Street vendor	Tomato	*Klebsiella pneumoniae*	2.452	2.444	+++	A	ESBL	Loandi Richter
UP_BN_LR_0232	UPMP 797	47	1B2 (F2)	Farmers' market	Green beans	*Klebsiella pneumoniae*	2.469	2.372	+++	A	ESBL	Loandi Richter

UP_BN_LR_0177	UPMP 742	48	A1 LT 4.1 B	Street vendor	Tomato	*Enterobacter asburiae*	1,97	2,044	+	B	ESBL	Loandi Richter
UP_BN_LR_0137	UPMP 702	49	A1 MTT 4.1 Ox	Mobile trolly	Tomato	*Klebsiella oxytoca*	1,91	1,962	+	C	ESBL	Loandi Richter
UP_BN_LR_0144	UPMP 709	50	F2 MT2 T1	Mobile trolly	Tomato	*Kluyvera ascorbata*	1,917	1,931	+	B	ESBL	Loandi Richter
UP_BN_LR_0210	UPMP 775	51	D4.1 RT4 T1	Retailer	Tomato	*Kluyvera ascorbata*	2,074	2,42	+++	A	ESBL	Loandi Richter
UP_BN_LR_0225	UPMP 790	52	GG2T1 (I14)	Street vendor	Tomato	*Proteus mirabilis*	2.518	2.488	+++	A	ESBL	Loandi Richter
UP_BN_LR_0231	UPMP 796	53	MT1T1 (I24)	Mobile trolly	Tomato	*Proteus mirabilis*	2.575	2.48	+++	A	ESBL	Loandi Richter
UP_BN_LR_0199	UPMP 764	54	C4.1 RT2 S4	Retailer	Spinach	*Proteus penneri*	2,302	2,282	++	A	ESBL	Loandi Richter
UP_BN_LR_0198	UPMP 763	55	C4.1 RT1 T2	Retailer	Tomato	*Proteus penneri*	2,19	2,147	++	C	ESBL	Loandi Richter
UP_BN_LR_0188	UPMP 753	56	E4 GG5 S4	Street vendor	Spinach	*Rahnella aquatilis*	2,06	2,206	++	A	ESBL	Loandi Richter
UP_BN_LR_0214	UPMP 779	57	D4 RT5 S2	Retailer	Spinach	*Rahnella aquatilis*	2,305	2,342	+++	A	ESBL	Loandi Richter
UP_BN_LR_0222	UPMP 787	58	3C3 (3.17)	Farmers' market	Cucumber	*Rahnella aquatilis*	2.399	1.95	+++	C	ESBL	Loandi Richter
UP_BN_LR_0227	UPMP 792	59	GG3T3 (I64)	Street vendor	Tomato	*Rahnella aquatilis*	2.071	2.013	++	C	ESBL	Loandi Richter
UP_BN_LR_0229	UPMP 794	60	GG4S3 (I73)	Street vendor	Spinach	*Rahnella aquatilis*	2.062	1.962	++	C	ESBL	Loandi Richter
UP_BN_LR_0265	UPMP 830	61	RT4T4 (R29)	Retailer	Tomato	*Rahnella aquatilis*	2.031	2.02	++	C	ESBL	Loandi Richter
UP_BN_LR_0209	UPMP 774	62	D4 RT4 T3	Retailer	Tomato	*Raoultella ornithinolytica*	2.613	2.538	+++	A	ESBL	Loandi Richter
UP_BN_LR_0181	UPMP 746	63	E4.1 GG5 S4	Street vendor	Spinach	*Serratia fonticola*	2.544	2.495	+++	A	ESBL	Loandi Richter
UP_BN_LR_0223	UPMP 788	64	4S1.1 (3.19)	Farmers' market	Spinach	*Serratia fonticola*	2.341	2.022	+++	A	ESBL	Loandi Richter
UP_BN_LR_0187	UPMP 752	65	E4.1 GG5 T3	Street vendor	Tomato	*Serratia fonticola*	2.453	2.438	+++	A	ESBL	Loandi Richter
UP_BN_LR_0251	UPMP 816	66	5B4 (F105)	Farmers' market	Green beans	*Serratia fonticola*	2.451	2.411	+++	A	ESBL	Loandi Richter
UP_BN_LR_0233	UPMP 798	67	4S1 (F60)	Farmers' market	Spinach	*Serratia fonticola*	2.231	2.056	++	A	ESBL	Loandi Richter
UP_BN_LR_0237	UPMP 802	68	4L2 (F75)	Farmers' market	Lettuce	*Serratia fonticola*	2.238	2.074	++	A	ESBL	Loandi Richter
UP_BN_LR_0238	UPMP 803	69	4L4 (F77)	Farmers' market	Lettuce	*Serratia fonticola*	2.343	2.17	+++	A	ESBL	Loandi Richter
UP_BN_LR_0239	UPMP 804	70	4L5 (F78)	Farmers' market	Lettuce	*Serratia fonticola*	2.369	2.227	+++	A	ESBL	Loandi Richter
UP_BN_LR_0241	UPMP 806	71	4L4 (F81)	Farmers' market	Lettuce	*Serratia fonticola*	2.285	2.114	++	A	ESBL	Loandi Richter
UP_BN_LR_0260	UPMP 825	72	RT2S1 (R8)	Retailer	Spinach	*Serratia fonticola*	1.875	1.859	+	B	ESBL	Loandi Richter
UP_BN_LR_0261	UPMP 826	73	RT2S2 (R9)	Retailer	Spinach	*Serratia fonticola*	2.253	2.113	++	A	ESBL	Loandi Richter
UP_BN_LR_0262	UPMP 827	74	RT2S4 (R11)	Retailer	Spinach	*Serratia fonticola*	2.058	1.902	++	A	ESBL	Loandi Richter
UP_BN_LR_0230	UPMP 795	75	GG5S1 (I81)	Street vendor	Spinach	*Serratia fonticola*	2.286	2.039	++	A	ESBL	Loandi Richter
UP_BN_LR_0228	UPMP 793	76	GG3S5 (I70)	Street vendor	Spinach	*Serratia fonticola*	2.228	2.11	++	A	ESBL	Loandi Richter
UP_BN_LR_0257	UPMP 822	77	5L2 (Fb)	Farmers' market	Lettuce	*Serratia marsecens*	2.283	2.153	++	A	ESBL	Loandi Richter

Appendix D

Table D1: Enterobacteriaceae, coliforms and *Escherichia coli* enumerated in water samples from a spinach production system where river water was used for irrigation

		Production scenario 1								
		Farm A			Farm A			Farm A		
Source	**Trip**	**Enterobacteriaceae (log CFU/ml)**			**Coliforms (log MPN/100ml)**			***Escherichia coli* (log MPN/100m)**		
		Range	**Mean ± SE[a]**	**t-Test[b]**	**Range**	**Mean ± SE[a]**	**t-Test[b]**	**Range**	**Mean ± SE[a]**	**t-Test[b]**
River	1	2,84 - 2,95	2,88 ± 0,04	AB	3,38 - 4,38	3,92 ± 0,29	B	2,20 - 2,48	2,29 ± 0,09	A
	2	3,04 - 3,20	3,11 ± 0,05	A	4,52 - 4,76	4,63 ± 0,07	A	2,38 - 2,64	2,52 ± 0,08	A
Dam (Reservoir)	1	1,61 - 3,78	2,72 ± 0,63	AB	3,19 - 3,38	3,32 ± 0,06	C	1,43 - 1,50	1,47 ± 0,02	B
	2	-	-	-	-	-	-	-	-	-
Irrigation pivot point	1	0,00 - 3,83	2,52 ± 1,26	B	3,11 - 3,19	3,17 ± 0,03	C	1,50 - 1,59	1,55 ± 0,02	B
	2	0,00 - 3,15	1,95 ± 0,98	C	4,51 - 4,76	4,59 ± 0,08	A	2,37 - 2,56	2,49 ± 0,06	A
Pack house dam	1	0,00 - 0,00	0,00 ± 0,00	-	0,00 - 0,00	0,00 ± 0,00	-	0,00 - 0,00	0,00 ± 0,00	-
	2	0,00 - 0,00	0,00 ± 0,00	-	0,00 - 0,00	0,00 ± 0,00	-	0,00 - 0,00	0,00 ± 0,00	-
Bunch wash basin	1	0,00 - 0,00	0,00 ± 0,00	D	0,00 - 0,61	0,30 ± 0,18	E	0,00 - 0,00	0,00 ± 0,00	D
	2	0,00 - 1,04	0,35 ± 0,35	D	1,58 - 1,99	1,78 ± 0,12	D	0,00 - 1,03	0,58 ± 0,31	C
Wash water	1	0,00 - 0,00	0,00 ± 0,00	D	0,00 - 0,00	0,00 ± 0,00	E	0,00 - 0,00	0,00 ± 0,00	D
	2	0,00 - 0,00	0,00 ± 0,00	D	0,00 - 0,30	0,10 ± 0,10	E	0,00 - 0,00	0,00 ± 0,00	D
p-value (source)				0,0083			<0,0001			<0,0001
p-value (trip)				0,9843			<0,0001			0,0012
p-value (trip x source)				0,0936			0,0077			0,0257

[a]SE: Standard error

[b]Within each column, means (based on the trip x source interactions) followed by the same letters are not significantly different ($p < 0,05$).

Appendix D

Table D2: Enterobacteriaceae, coliforms and *Escherichia coli* enumerated in spinach samples from a spinach production system where river water was used for irrigation

		Production scenario 1								
		Farm A			Farm A			Farm A		
Source	Trip	Enterobacteriaceae (log CFU/g)			Coliforms (log CFU/g)			*Escherichia coli* (log CFU/g)		
		Range	Mean ± SE[a]	t-Test[b]	Range	Mean ± SE[a]	t-Test[b]	Range	Mean ± SE[a]	t-Test[b]
Spinach at Harvest	1	5,54 - 6,16	5,88 ± 0,10	A	5,52 - 6,05	5,73 ± 0,10	AB	0,00 - 0,00	0,00 ± 0,00	C
	2	4,64 - 5,65	5,00 ± 0,18	A	4,12 - 4,99	4,54 ± 0,16	E	0,00 - 2,18	0,78 ± 0,48	BC
Spinach at receival (packhouse)	1	0,00 - 6,41	4,06 ± 2,04	A	4,38 - 6,50	5,46 ± 0,61	ABC	0,00 - 0,00	0,00 ± 0,00	C
	2	4,37 - 4,71	4,49 ± 0,11	A	4,12 - 5,51	4,67 ± 0,42	DE	1,71 - 4,03	3,22 ± 0,76	A
Spinach bunches at dispatch (packhouse)	1	5,79 - 5,80	5,80 ± 0,00	A	5,59 - 6,39	5,89 ± 0,25	A	0,00 - 0,00	0,00 ± 0,00	C
	2	0,00 - 6,02	3,61 ± 1,84	A	4,10 - 5,60	4,94 ± 0,44	CDE	0,00 - 0,00	0,00 ± 0,00	C
Spinach after cut	1	6,03 - 6,20	6,09 ± 0,05	A	5,47 - 5,59	5,55 ± 0,04	ABC	0,00 - 0,00	0,00 ± 0,00	C
	2	5,73 - 5,81	5,77 ± 0,02	A	5,46 - 5,63	5,57 ± 0,05	ABC	0,00 - 2,30	1,34 ± 0,69	B
Spinach after wash	1	4,92 - 5,35	5,18 ± 0,13	A	3,90 - 5,65	4,54 ± 0,56	E	0,00 - 0,00	0,00 ± 0,00	C
	2	5,04 - 5,86	5,33 ± 0,26	A	5,27 - 5,51	5,40 ± 0,07	ABCD	0,00 - 0,00	0,00 ± 0,00	C
Spinach after pack	1	4,60 - 5,28	5,04 ± 0,22	A	4,81 - 5,10	4,98 ± 0,09	BCDE	0,00 - 0,00	0,00 ± 0,00	C
	2	5,82 - 6,07	5,90 ± 0,08	A	5,77 - 6,15	6,00 ± 0,12	A	0,00 - 2,30	1,49 ± 0,75	B
Spinach at Retailer	1	4,78 - 5,84	5,38 ± 0,18	A	5,21 - 5,80	5,39 ± 0,11	ABCD	0,00 - 0,00	0,00 ± 0,00	C
	2	5,92 - 6,52	6,17 ± 0,10	A	5,75 - 6,33	6,16 ± 0,11	A	0,00 - 1,71	0,34 ± 0,34	C
Spinach bunches at retailer	1	5,22 - 6,16	5,66 ± 0,16	A	5,41 - 5,96	5,70 ± 0,10	ABC	0,00 - 0,00	0,00 ± 0,00	C
	2	4,37 - 5,30	4,89 ± 0,18	A	3,95 - 4,92	4,55 ± 0,22	E	0,00 - 0,00	0,00 ± 0,00	C
p-value (source)				0,1646			0,0215			0,0012
p-value (trip)				0,3639			0,1412			<0,0001
p-value (trip x source)				0,1627			0,0003			0,0012

[a]SE: Standard error

[b]Within each column, means (based on the trip x source interactions) followed by the same letters are not significantly different ($p < 0,05$).

Table D3: Enterobacteriaceae, coliforms and *Escherichia coli* enumerated in contact surface samples from a spinach production system where river water was used for irrigation

		Production scenario 1								
		Farm A			Farm A			Farm A		
Source	**Trip**	**Enterobacteriaceae (log CFU/cm^2)**			**Coliforms (log CFU/cm^2)**			***Escherichia coli* (log CFU/cm^2)**		
		Range	**Mean ± SE[a]**	**t-Test[b]**	**Range**	**Mean ± SE[a]**	**t-Test[b]**	**Range**	**Mean ± SE[a]**	**t-Test[b]**
Crates	1	5,03 - 5,33	5,14 ± 0,10	AB	4,53 - 5,15	4,79 ± 0,18	AB	0,00 - 0,00	0,00 ± 0,00	B
	2	3,81 - 4,40	4,02 ± 0,19	AB	2,60 - 3,70	3,30 ± 0,35	D	0,00 - 2,00	1,21 ± 0,61	AB
Floors	1	4,04 - 5,48	4,53 ± 0,48	AB	3,31 - 5,14	4,42 ± 0,56	BC	0,00 - 0,00	0,00 ± 0,00	B
	2	4,35 - 6,13	4,99 ± 0,57	AB	4,98 - 6,32	5,57 ± 0,39	A	1,32 - 2,74	2,09 ± 0,41	A
Cutting surfaces	1	4,99 - 5,67	5,27 ± 0,20	A	5,00 - 5,87	5,36 ± 0,26	A	0,00 - 0,00	0,00 ± 0,00	B
	2	2,70 - 4,11	3,56 ± 0,44	B	3,38 - 4,30	3,96 ± 0,28	CD	0,00 - 1,79	0,94 ± 0,40	AB
p-value (source)				0,4228			0,1838			0,3326
p-value (trip)				0,0853			0,0222			0,0034
p-value (trip x source)				0,1333			0,0021			0,3326

[a]SE: Standard error

[b]Within each column, means (based on the trip x source interactions) followed by the same letters are not significantly different ($p < 0,05$).

[c]Within each column, means (based on the trip interactions) followed by the same letters are not significantly different ($p < 0,05$).

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Table D4: Enterobacteriaceae, coliforms and *Escherichia coli* enumerated in water samples from a spinach production system where borehole water was used for irrigation

		Production scenario 2								
		Farm B			Farm B			Farm B		
Source	Trip	Enterobacteriaceae (log CFU/ml)			Coliforms (log MPN/100ml)			*Escherichia coli* (log MPN/100ml)		
		Range	Mean ± SE[a]	t-Test[b]	Range	Mean ± SE[a]	t-Test[b]	Range	Mean ± SE[a]	t-Test[b]
Dam (Source)	1	0,00 - 0,00	0,00 ± 0,00	C	0,00 - 0,00	0,00 ± 0,00	D	0,00 - 0,00	0,00 ± 0,00	D
	2	0,00 - 0,00	0,00 ± 0,00	C	0,00 - 0,00	0,00 ± 0,00	D	0,00 - 0,00	0,00 ± 0,00	D
Dam (Reservoir)	1	0,78 - 1,71	1,23 ± 0,27	B	2,65 - 2,74	2,71 ± 0,03	BC	0,61 - 1,04	0,84 ± 0,12	B
	2	2,45 - 2,46	2,46 ± 0,00	A	3,66 - 3,84	3,77 ± 0,05	A	4,24 - 4,56	4,40 ± 0,09	A
Irrigation pivot point	1	0,00 - 1,85	1,09 ± 0,56	B	2,35 - 2,64	2,45 ± 0,09	C	0,30 - 0,72	0,50 ± 0,12	C
	2	2,26 - 2,49	2,36 ± 0,05	A	2,71 - 3,64	3,09 ± 0,28	B	0,00 - 0,00	0,00 ± 0,00	D
Wash water	1	0,00 - 0,00	0,00 ± 0,00	C	0,00 - 0,00	0,00 ± 0,00	D	0,00 - 0,00	0,00 ± 0,00	D
	2	0,00 - 0,00	0,00 ± 0,00	C	0,00 - 0,00	0,00 ± 0,00	D	0,00 - 0,00	0,00 ± 0,00	D
p-value (source)				<0,0001			<0,0001			<0,0001
p-value (trip)				0,0058			0,0015			<0,0001
p-value (trip x source)				0,0365			0,0074			<0,0001

[a]SE: Standard error

[b]Within each column, means (based on the trip x source interactions) followed by the same letters are not significantly different ($p < 0,05$).

Table D5: Enterobacteriaceae, coliforms and *Escherichia coli* enumerated in baby spinach samples from a spinach production system where borehole water was used for irrigation

		Production scenario 2								
		Farm B			Farm B			Farm B		
Source	Trip	Enterobacteriaceae (log CFU/g)			Coliforms (log CFU/g)			*Escherichia coli* (log CFU/g)		
		Range	Mean ± SE[a]	t-Test[b]	Range	Mean ± SE[a]	t-Test[b]	Range	Mean ± SE[a]	t-Test[b]
Spinach at Harvest	1	3,28 - 4,93	4,10 ± 0,26	CDE	0,00 - 4,72	3,33 ± 0,87	G	0,00 - 2,00	0,40 ± 0,40	A
	2	5,08 - 5,50	5,34 ± 0,08	AB	4,90 - 5,48	5,16 ± 0,12	BCD	0,00 - 0,00	0,00 ± 0,00	A
Spinach at Dispatch (crates)	1	3,65 - 6,04	4,46 ± 0,79	BCDE	3,08 - 5,86	4,03 ± 0,91	DEFG	0,00 - 0,00	0,00 ± 0,00	A
	2	4,12 - 5,08	4,74 ± 0,24	BC	4,57 - 5,02	4,73 ± 0,14	CDE	0,00 - 0,00	0,00 ± 0,00	A
Spinach punnets at dispatch (packhouse)	1	0,00 - 7,05	4,56 ± 2,28	BCD	6,43 - 6,65	6,57 ± 0,07	A	0,00 - 0,00	0,00 ± 0,00	A
	2	0,00 - 5,34	3,50 ± 1,36	DEF	4,70 - 4,93	4,84 ± 0,07	BCDE	0,00 - 0,00	0,00 ± 0,00	A
Spinach at Receival (processing facility)	1	4,51 - 5,38	4,90 ± 0,25	BC	3,59 - 4,12	3,92 ± 0,16	EFG	0,00 - 0,00	0,00 ± 0,00	A
	2	5,34 - 5,95	5,56 ± 0,15	AB	4,94 - 5,68	5,20 ± 0,24	BCD	0,00 - 0,00	0,00 ± 0,00	A
Spinach punnets at receival (processing facility)	1	-	-	-	-	-	-	-	-	-
	2	5,09 - 5,75	5,35 ± 0,15	AB	4,72 - 5,61	5,05 ± 0,28	BCDE	0,00 - 0,00	0,00 ± 0,00	A
Spinach after wash	1	2,98 - 3,72	3,33 ± 0,21	EF	2,89 - 3,91	3,49 ± 0,31	FG	0,00 - 0,00	0,00 ± 0,00	A
	2	4,43 - 6,96	5,42 ± 0,61	AB	4,27 - 4,41	4,35 ± 0,04	CDEFG	0,00 - 0,00	0,00 ± 0,00	A
Spinach after pack	1	0,00 - 3,88	2,47 ± 1,24	F	3,08 - 3,91	3,39 ± 0,27	G	0,00 - 0,00	0,00 ± 0,00	A
	2	4,50 - 5,01	4,82 ± 0,13	BC	4,28 - 5,38	4,84 ± 0,32	BCDE	0,00 - 0,00	0,00 ± 0,00	A
Spinach at Retailer	1	5,22 - 5,59	5,37 ± 0,08	AB	4,94 - 5,82	5,43 ± 0,14	ABC	0,00 - 0,00	0,00 ± 0,00	A
	2	3,95 - 5,41	4,49 ± 0,28	BCDE	4,15 - 5,41	4,43 ± 0,24	CDEFG	0,00 - 0,00	0,00 ± 0,00	A
Spinach punnets at retailer	1	5,80 - 6,46	6,10 ± 0,14	A	5,56 - 6,38	5,99 ± 0,14	AB	0,00 - 0,00	0,00 ± 0,00	A
	2	3,92 - 5,82	5,14 ± 0,33	ABC	3,69 - 5,43	4,65 ± 0,28	CDEF	0,00 - 0,00	0,00 ± 0,00	A
p-value (source)				0,4192			0,0037			0,7439
p-value (trip)				0,1034			0,3915			0,3488

p-value (trip x source)	0,0006	0,0002	0,7069

[a]SE: Standard error

[b]Within each column, means (based on the trip x source interactions) followed by the same letters are not significantly different ($p < 0,05$).

Table D6: Enterobacteriaceae, coliforms and *Escherichia coli* enumerated in water samples from a spinach production system where borehole water was used for irrigation

		Production scenario 2								
		Farm C			**Farm C**			**Farm C**		
Source	**Trip**	**Enterobacteriaceae (log CFU/ml)**			**Coliforms (log MPN/100ml)**			***Escherichia coli* (log MPN/100ml)**		
		Range	**Mean ± SE[a]**	**t-Test[b]**	**Range**	**Mean ± SE[a]**	**t-Test[b]**	**Range**	**Mean ± SE[a]**	**t-Test[b]**
Dam (Source)	1	3,19 - 3,23	3,21 ± 0,01	A	5,09 - 5,44	5,24 ± 0,10	A	0,30 - 0,61	0,51 ± 0,10	A
	2	2,41 - 2,49	2,45 ± 0,02	B	4,44 - 4,48	4,46 ± 0,01	B	0,00 - 0,00	0,00 ± 0,00	B
Irrigation pivot point	1	1,20 - 1,71	1,41 ± 0,15	C	2,15 - 2,44	2,28 ± 0,09	C	0,00 - 0,00	0,00 ± 0,00	B
	2	0,00 - 0,00	0,00 ± 0,00	D	0,93 - 2,31	1,44 ± 0,44	D	0,00 - 0,00	0,00 ± 0,00	B
Wash water	1	0,00 - 0,00	0,00 ± 0,00	D	0,00 - 0,30	0,10 ± 0,10	E	0,00 - 0,00	0,00 ± 0,00	B
	2	0,00 - 0,00	0,00 ± 0,00	D	0,00 - 0,61	0,20 ± 0,20	E	0,00 - 0,00	0,00 ± 0,00	B
p-value (source)				<0,0001			<0,0001			0,0014
p-value (trip)				<0,0001			0,0166			0,0027
p-value (trip x source)				<0,0001			0,0804			0,0014

[a]SE: Standard error

[b]Within each column, means (based on the trip x source interactions) followed by the same letters are not significantly different ($p < 0,05$).

Table D7: Enterobacteriaceae, coliforms and *Escherichia coli* enumerated in baby spinach samples from a spinach production system where borehole water was used for irrigation

		Production scenario 2								
		Farm C			Farm C			Farm C		
Source	Trip	Enterobacteriaceae (log CFU/g)			Coliforms (log CFU/g)			*Escherichia coli* (log CFU/g)		
		Range	Mean ± SE[a]	t-Test[b]	Range	Mean ± SE[a]	t-Test[b]	Range	Mean ± SE[a]	t-Test[b]
Spinach at Harvest	1	3,51 - 4,18	3,92 ± 0,11	G	3,56 - 4,10	3,93 ± 0,10	D	0,00 - 0,00	0,00 ± 0,00	A
	2	3,99 - 7,07	6,03 ± 0,55	ABC	6,13 - 7,01	6,36 ± 0,35	AB	0,00 - 3,70	0,74 ± 0,74	A
Spinach at receival (packhouse)	1	0,00 - 5,26	3,32 ± 1,67	G	1,04 - 4,99	3,37 ± 1,19	D	0,00 - 1,71	0,57 ± 0,57	A
	2	0,00 - 5,42	3,33 ± 1,68	G	1,04 - 5,55	4,01 ± 1,48	D	0,00 - 1,71	0,57 ± 0,57	A
Spinach punnets at dispatch (packhouse)	1	5,75 - 6,74	6,17 ± 0,30	AB	5,82 - 6,54	6,11 ± 0,22	ABC	0,00 - 0,00	0,00 ± 0,00	A
	2	5,63 - 5,72	5,66 ± 0,03	BCDE	5,42 - 5,79	5,66 ± 0,12	BC	0,00 - 0,00	0,00 ± 0,00	A
Spinach at Receival (processing facility)	1	4,10 - 4,95	4,66 ± 0,28	F	3,94 - 4,16	4,06 ± 0,06	D	0,00 - 0,00	0,00 ± 0,00	A
	2	5,20 - 5,38	5,30 ± 0,05	DEF	5,40 - 6,69	6,03 ± 0,37	ABC	0,00 - 0,00	0,00 ± 0,00	A
Spinach punnets at receival (processing facility)	1	6,30 - 6,67	6,52 ± 0,11	A	3,94 - 4,16	4,06 ± 0,06	E	0,00 - 0,00	0,00 ± 0,00	A
	2	5,20 - 5,46	5,32 ± 0,07	CDEF	1,04 - 5,55	4,01 ± 1,48	D	0,00 - 0,00	0,00 ± 0,00	A
Spinach after wash	1	3,23 - 3,43	3,35 ± 0,06	G	3,28 - 5,26	4,06 ± 0,61	D	0,00 - 0,00	0,00 ± 0,00	A
	2	4,66 - 5,14	4,95 ± 0,15	EF	5,71 - 5,98	5,87 ± 0,08	ABC	0,00 - 0,00	0,00 ± 0,00	A
Spinach after pack	1	3,75 - 3,95	3,84 ± 0,06	G	3,69 - 4,00	3,86 ± 0,09	D	0,00 - 0,00	0,00 ± 0,00	A
	2	5,03 - 5,81	5,48 ± 0,23	BCDE	5,66 - 6,07	5,80 ± 0,14	ABC	0,00 - 0,00	0,00 ± 0,00	A
Spinach at Retailer	1	3,56 - 4,01	3,72 ± 0,07	G	3,73 - 3,98	3,84 ± 0,05	D	0,00 - 0,00	0,00 ± 0,00	A
	2	4,93 - 5,80	5,27 ± 0,15	DEF	4,91 - 5,80	5,35 ± 0,17	C	0,00 - 0,00	0,00 ± 0,00	A
Spinach punnets at retailer	1	6,33 - 6,78	6,57 ± 0,09	A	6,34 - 6,85	6,64 ± 0,10	A	0,00 - 0,00	0,00 ± 0,00	A
	2	5,50 - 6,53	5,90 ± 0,19	ABCD	5,47 - 6,05	5,73 ± 0,10	ABC	0,00 - 2,00	0,80 ± 0,49	A
p-value (source)				0,0042			0,0006			0,6275

p-value (trip)	<0,0001	<0,0001	0,1109
p-value (trip x source)	<0,0001	<0,0001	0,6166

Table D8: Enterobacteriaceae, coliforms and *Escherichia coli* enumerated in contact surface samples from a spinach production system where borehole water was used for irrigation

		Production scenario 2								
		Farm C			Farm C			Farm C		
Source	**Trip**	**Enterobacteriaceae (log CFU/cm²)**			**Coliforms (log CFU/cm²)**			***Escherichia coli* (log CFU/cm²)**		
		Range	**Mean ± SE[a]**	**t-Test[c]**	**Range**	**Mean ± SE[a]**	**t-Test[c]**	**Range**	**Mean ± SE[a]**	**t-Test[c]**
Cutting surfaces	1	2,18 - 3,60	2,85 ± 0,41	A	0,00 - 2,72	0,91 ± 0,91	A	0,00 - 0,00	0,00 ± 0,00	A
	2	5,15 - 6,10	5,71 ± 0,29	B	4,81 - 5,00	4,93 ± 0,06	B	0,00 - 0,00	0,00 ± 0,00	A
p-value (source)				-			-			-
p-value (trip)				0,0333			0,045			-
p-value (trip x source)				-			-			-

[a]SE: Standard error

[b]Within each column, means (based on the trip x source interactions) followed by the same letters are not significantly different ($p < 0,05$).

[c]Within each column, means (based on the trip interactions) followed by the same letters are not significantly different ($p < 0,05$).

Table D9: Enterobacteriaceae, coliforms and *Escherichia coli* enumerated from soil samples during harvest on three farms representing two spinach production scenarios

Farm	Trip	Enterobacteriaceae (log CFU/g)	Coliforms (log CFU/g)	*Escherichia coli* (log CFU/g)
		Mean ± SE[a]	Mean ± SE[a]	Mean ± SE[a]
A	1	5,02	4,68	0
	2	5,22	5,19	0
B	1	3,88	3,60	0
	2	3,29	3,05	0
C	1	4,08	3,84	0
	2	4,07	4,08	0

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Table D10: Matrix-assisted laser desorption time-of-flight identification of *Escherichia coli* isolated from commercial spinach production systems in Gauteng Province

University of Pretoria Culture number	UPMP code	Sample Code	Farm/Vendor	Trip	Source	Presumptive Organism Identity	Organism best match*		Range**	Consistency category***	Notes	Isolated by
							Score 1	Score 2				
UP_BN_LR_0001	UPMP 566	A1 MPi3	Farm A	Trip 1	Pivot Point	*Escherichia coli*	2.478	2.444	+++	A	Generic	Loandi Richter
UP_BN_LR_0002	UPMP 567	A1 MR1	Farm A	Trip 1	Water reservoir	*Escherichia coli*	2.399	2.384	+++	A	Generic	Loandi Richter
UP_BN_LR_0003	UPMP 568	A1 MR2	Farm A	Trip 1	Water reservoir	*Escherichia coli*	2.475	2.448	+++	A	Generic	Loandi Richter
UP_BN_LR_0004	UPMP 569	A1 MR3	Farm A	Trip 1	Water reservoir	*Escherichia coli*	2.412	2.401	+++	A	Generic	Loandi Richter
UP_BN_LR_0005	UPMP 570	A2 Pi1.1	Farm A	Trip 2	Pivot Point	*Escherichia coli*	2.557	2.553	+++	A	Generic	Loandi Richter
UP_BN_LR_0006	UPMP 571	A2 Pi2.1	Farm A	Trip 2	Pivot Point	*Escherichia coli*	2.585	2.485	+++	A	Generic	Loandi Richter
UP_BN_LR_0007	UPMP 572	A2 Pi3.1	Farm A	Trip 2	Pivot Point	*Escherichia coli*	2.536	2.522	+++	A	Generic	Loandi Richter
UP_BN_LR_0008	UPMP 573	A2 R1.1	Farm A	Trip 2	Water reservoir	*Escherichia coli*	2.423	2.418	+++	A	Generic	Loandi Richter
UP_BN_LR_0009	UPMP 574	A2 R1.2	Farm A	Trip 2	Water reservoir	*Escherichia coli*	2.45	2.415	+++	A	Generic	Loandi Richter
UP_BN_LR_0010	UPMP 575	A2 R1.3	Farm A	Trip 2	Water reservoir	*Escherichia coli*	2.465	2.346	+++	A	Generic	Loandi Richter
UP_BN_LR_0011	UPMP 576	A2 R2	Farm A	Trip 2	Water reservoir	*Escherichia coli*	2.486	2.43	+++	A	Generic	Loandi Richter
UP_BN_LR_0012	UPMP 577	A2 R2.1	Farm A	Trip 2	Water reservoir	*Escherichia coli*	2.553	2.463	+++	A	Generic	Loandi Richter
UP_BN_LR_0013	UPMP 578	A2 R2.2	Farm A	Trip 2	Water reservoir	*Escherichia coli*	2.431	2.364	+++	A	Generic	Loandi Richter
UP_BN_LR_0014	UPMP 579	A2 R2.3	Farm A	Trip 2	Water reservoir	*Escherichia coli*	2.331	2.33	+++	A	Generic	Loandi Richter
UP_BN_LR_0015	UPMP 580	A2 R3	Farm A	Trip 2	Water reservoir	*Escherichia coli*	2.509	2.473	+++	A	Generic	Loandi Richter
UP_BN_LR_0016	UPMP 581	A2 R3.1	Farm A	Trip 2	Water reservoir	*Escherichia coli*	2.415	2.393	+++	A	Generic	Loandi Richter
UP_BN_LR_0017	UPMP 582	A2 R3.2	Farm A	Trip 2	Water reservoir	*Escherichia coli*	2.517	2.453	+++	A	Generic	Loandi Richter
UP_BN_LR_0018	UPMP 583	A2 R3.3	Farm A	Trip 2	Water reservoir	*Escherichia coli*	2.543	2.508	+++	A	Generic	Loandi Richter
UP_BN_LR_0019	UPMP 584	A2 Sp1URe	Farm A	Trip 2	Unwashed spinach at receival	*Escherichia coli*	2.613	2.538	+++	A	Generic	Loandi Richter
UP_BN_LR_0020	UPMP 585	A2 Sp2RT	Farm A	Trip 2	Spinach at retailer	*Escherichia coli*	2.544	2.495	+++	A	Generic	Loandi Richter

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UP_BN_LR_0021	UPMP 586	A2 Sp3URT	Farm A	Trip 2	Unwashed spinach punnets at retailer	*Escherichia coli*	2.401	2.362	+++	A	Generic	Loandi Richter
UP_BN_LR_0022	UPMP 587	A2 Sp4RT	Farm A	Trip 2	Spinach at retailer	*Escherichia coli*	2.545	2.492	+++	A	Generic	Loandi Richter
UP_BN_LR_0023	UPMP 588	ESBL A2 R1	Farm A	Trip 2	Water reservoir	*Escherichia coli*	2.544	2.495	+++	A	ESBL	Loandi Richter
UP_BN_LR_0024	UPMP 589	ESBL A2 R2	Farm A	Trip 2	Water reservoir	*Escherichia coli*	2,495	2,46	+++	A	ESBL	Loandi Richter
UP_BN_LR_0025	UPMP 590	ESBL A2 R3	Farm A	Trip 2	Water reservoir	*Escherichia coli*	2,47	2,383	+++	A	ESBL	Loandi Richter
UP_BN_LR_0026	UPMP 591	Sp3H1	Farm B	Trip 1	Spinach at harvest	*Escherichia coli*	2,433	2,365	+++	A	Generic	Loandi Richter
UP_BN_LR_0027	UPMP 592	Sp5H	Farm B	Trip1	Spinach at harvest	*Escherichia coli*	2.514	2.463	+++	A	Generic	Loandi Richter
UP_BN_LR_0028	UPMP 593	Sp2U	Farm B	Trip 1	Unwashed spinach bunches at dispatch	*Escherichia coli*	2.452	2.447	+++	A	Generic	Loandi Richter
UP_BN_LR_0029	UPMP 594	Sp2URT	Farm B	Trip 1	Unwashed spinach bunches at retailer	*Escherichia coli*	2.336	2.266	+++	A	Generic	Loandi Richter
UP_BN_LR_0030	UPMP 595	Sp3URT	Farm B	Trip 1	Unwashed spinach bunches at retailer	*Escherichia coli*	2.444	2.435	+++	A	Generic	Loandi Richter
UP_BN_LR_0032	UPMP 597	R 1	Farm B	Trip 1	River water	*Escherichia coli*	2.494	2.467	+++	A	Generic	Loandi Richter
UP_BN_LR_0033	UPMP 598	R 2	Farm B	Trip 1	River water	*Escherichia coli*	2.544	2.498	+++	A	Generic	Loandi Richter
UP_BN_LR_0034	UPMP 599	D2	Farm B	Trip 1	Holding dam water	*Escherichia coli*	2.447	2.439	+++	A	Generic	Loandi Richter
UP_BN_LR_0035	UPMP 600	D3	Farm B	Trip 1	Holding dam water	*Escherichia coli*	2.576	2.531	+++	A	Generic	Loandi Richter
UP_BN_LR_0036	UPMP 601	Pi3	Farm B	Trip 1	Irrigation pivot point water	*Escherichia coli*	2.45	2.441	+++	A	Generic	Loandi Richter
UP_BN_LR_0037	UPMP 602	F3	Farm B	Trip 1	Floor in packhouse	*Escherichia coli*	2.567	2.507	+++	A	Generic	Loandi Richter
UP_BN_LR_0038	UPMP 603	Sp2H	Farm B	Trip 2	Spinach at harvest	*Escherichia coli*	2.485	2.423	+++	A	Generic	Loandi Richter
UP_BN_LR_0039	UPMP 604	Sp4H	Farm B	Trip 2	Spinach at harvest	*Escherichia coli*	2.517	2.504	+++	A	Generic	Loandi Richter
UP_BN_LR_0040	UPMP 605	Sp3H2	Farm B	Trip 2	Spinach at harvest	*Escherichia coli*	2.573	2.488	+++	A	Generic	Loandi Richter
UP_BN_LR_0041	UPMP 606	Sp1U	Farm B	Trip 2	Unwashed spinach bunches at dispatch	*Escherichia coli*	2.46	2.434	+++	A	Generic	Loandi Richter
UP_BN_LR_0042	UPMP 607	Sp2Re1	Farm B	Trip 2	Spinach at receival (in packhouse)	*Escherichia coli*	2.555	2.506	+++	A	Generic	Loandi Richter
UP_BN_LR_0043	UPMP 608	Sp2AC	Farm B	Trip 2	Spinach after cut	*Escherichia coli*	2.582	2.532	+++	A	Generic	Loandi Richter
UP_BN_LR_0044	UPMP 609	Sp3AW	Farm B	Trip 2	Spinach after wash	*Escherichia coli*	2.569	2.552	+++	A	Generic	Loandi Richter
UP_BN_LR_0045	UPMP 610	ESBL Sp3AC	Farm B	Trip 2	Spinach after cut	*Escherichia coli*	2,464	2,407	+++	A	ESBL	Loandi Richter

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UP_BN_LR_0046	UPMP 611	Sp3URT	Farm B	Trip 2	Unwashed spinach bunches at retailer	*Escherichia coli*	2.526	2.521	+++	A	Generic	Loandi Richter
UP_BN_LR_0048	UPMP 613	R 1	Farm B	Trip 2	River water	*Escherichia coli*	2.544	2.476	+++	A	Generic	Loandi Richter
UP_BN_LR_0049	UPMP 614	R 2	Farm B	Trip 2	River water	*Escherichia coli*	2.544	2.476	+++	A	Generic	Loandi Richter
UP_BN_LR_0050	UPMP 615	R 3	Farm B	Trip 2	River water	*Escherichia coli*	2.601	2.568	+++	A	Generic	Loandi Richter
UP_BN_LR_0051	UPMP 616	ESBL R1	Farm B	Trip 2	River water	*Escherichia coli*	2,475	2,443	+++	A	ESBL	Loandi Richter
UP_BN_LR_0052	UPMP 617	Pi3	Farm B	Trip 2	Irrigation pivot point water	*Escherichia coli*	2.533	2.487	+++	A	Generic	Loandi Richter
UP_BN_LR_0053	UPMP 618	Pi2	Farm B	Trip 2	Irrigation pivot point water	*Escherichia coli*	2.531	2.501	+++	A	Generic	Loandi Richter
UP_BN_LR_0054	UPMP 619	Pi1	Farm B	Trip 2	Irrigation pivot point water	*Escherichia coli*	2.501	2.404	+++	A	Generic	Loandi Richter
UP_BN_LR_0056	UPMP 621	ESBL Pi1,1	Farm B	Trip 2	Irrigation pivot point water	*Escherichia coli*	2,375	2,359	+++	A	ESBL	Loandi Richter
UP_BN_LR_0057	UPMP 622	S	Farm B	Trip 2	Soil	*Escherichia coli*	2.539	2.525	+++	A	Generic	Loandi Richter
UP_BN_LR_0058	UPMP 623	C2	Farm B	Trip 2	Crates	*Escherichia coli*	2.585	2.51	+++	A	Generic	Loandi Richter
UP_BN_LR_0059	UPMP 624	C3	Farm B	Trip 2	Crates	*Escherichia coli*	2.501	2.431	+++	A	Generic	Loandi Richter
UP_BN_LR_0060	UPMP 625	CS2,1	Farm B	Trip 2	Crates	*Escherichia coli*	2.445	2.437	+++	A	Generic	Loandi Richter
UP_BN_LR_0061	UPMP 626	CS3,1	Farm B	Trip 2	Crates	*Escherichia coli*	2.609	2.532	+++	A	Generic	Loandi Richter
UP_BN_LR_0062	UPMP 627	F1,2	Farm B	Trip 2	Floor in packhouse	*Escherichia coli*	2.44	2.413	+++	A	Generic	Loandi Richter
UP_BN_LR_0063	UPMP 628	F2,1	Farm B	Trip 2	Floor in packhouse	*Escherichia coli*	2.6	2.543	+++	A	Generic	Loandi Richter
UP_BN_LR_0069	UPMP 634	Sp2H	Farm C	Trip 1	Spinach at harvest	*Escherichia coli*	2.543	2.496	+++	A	Generic	Loandi Richter
UP_BN_LR_0070	UPMP 635	Sp1H	Farm C	Trip 1	Spinach at harvest	*Escherichia coli*	2.522	2.455	+++	A	Generic	Loandi Richter
UP_BN_LR_0071	UPMP 636	JJSp1Re	Farm C	Trip 1	Spinach at receival (packhouse)	*Escherichia coli*	2.59	2.578	+++	A	Generic	Loandi Richter
UP_BN_LR_0072	UPMP 637	JJSp2Re	Farm C	Trip 1	Spinach at receival (packhouse)	*Escherichia coli*	2.568	2.5	+++	A	Generic	Loandi Richter
UP_BN_LR_0073	UPMP 638	JJSp3Re	Farm C	Trip 1	Spinach at receival (packhouse)	*Escherichia coli*	2.57	2.507	+++	A	Generic	Loandi Richter
UP_BN_LR_0074	UPMP 639	ZFD1,1	Farm C	Trip 1	Holding dam water (source water)	*Escherichia coli*	2.569	2.528	+++	A	Generic	Loandi Richter
UP_BN_LR_0075	UPMP 640	ZFD1,2	Farm C	Trip 1	Holding dam water (source water)	*Escherichia coli*	2.465	2.437	+++	A	Generic	Loandi Richter
UP_BN_LR_0076	UPMP 641	ZFD3,1	Farm C	Trip 1	Holding dam water (source water)	*Escherichia coli*	2.678	2.532	+++	A	Generic	Loandi Richter

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UP_BN_LR_0077	UPMP 642	ZFD3,2	Farm C	Trip 1	Holding dam water (source water)	*Escherichia coli*	2.532	2.413	+++	A	Generic	Loandi Richter
UP_BN_LR_0078	UPMP 643	Sp3URT	Farm C	Trip 2	Unwashed spinach punnets at retailer	*Escherichia coli*	2.436	2.375	+++	A	Generic	Loandi Richter
UP_BN_LR_0079	UPMP 644	Sp3URT1	Farm C	Trip 2	Unwashed spinach punnets at retailer	*Escherichia coli*	2.533	2.415	+++	A	Generic	Loandi Richter
UP_BN_LR_0080	UPMP 645	Sp2URT	Farm C	Trip 2	Unwashed spinach punnets at retailer	*Escherichia coli*	2.599	2.555	+++	A	Generic	Loandi Richter
UP_BN_LR_0081	UPMP 646	Sp4RT	Farm C	Trip 2	Spinach at retailer	*Escherichia coli*	2.497	2.39	+++	A	Generic	Loandi Richter
UP_BN_LR_0082	UPMP 647	JJSp2U	Farm C	Trip 2	Unwashed spinach at dispatch (packhouse)	*Escherichia coli*	2.392	2.362	+++	A	Generic	Loandi Richter
UP_BN_LR_0083	UPMP 648	D1	Farm C	Trip 2	Holding dam water (source water)	*Escherichia coli*	2.497	2.447	+++	A	Generic	Loandi Richter
UP_BN_LR_0084	UPMP 649	D2	Farm C	Trip 2	Holding dam water (source water)	*Escherichia coli*	2.528	2.454	+++	A	Generic	Loandi Richter
UP_BN_LR_0085	UPMP 650	D3,1	Farm C	Trip 2	Holding dam water (source water)	*Escherichia coli*	2.501	2.42	+++	A	Generic	Loandi Richter
UP_BN_LR_0086	UPMP 651	ESBL Sp4RT	Farm C	Trip 2	Spinach at retailer	*Escherichia coli*	2,476	2,552	+++	A	ESBL	Loandi Richter
UP_BN_LR_0087	UPMP 652	ESBL JJSp2U	Farm C	Trip 2	Unwashed spinach at dispatch (packhouse)	*Escherichia coli*	2,525	2,523	+++	A	ESBL	Loandi Richter
UP_BN_LR_0088	UPMP 653	ESBL D1	Farm C	Trip 2	Holding dam water (source water)	*Escherichia coli*	2,446	2,63	+++	A	ESBL	Loandi Richter

Appendix E

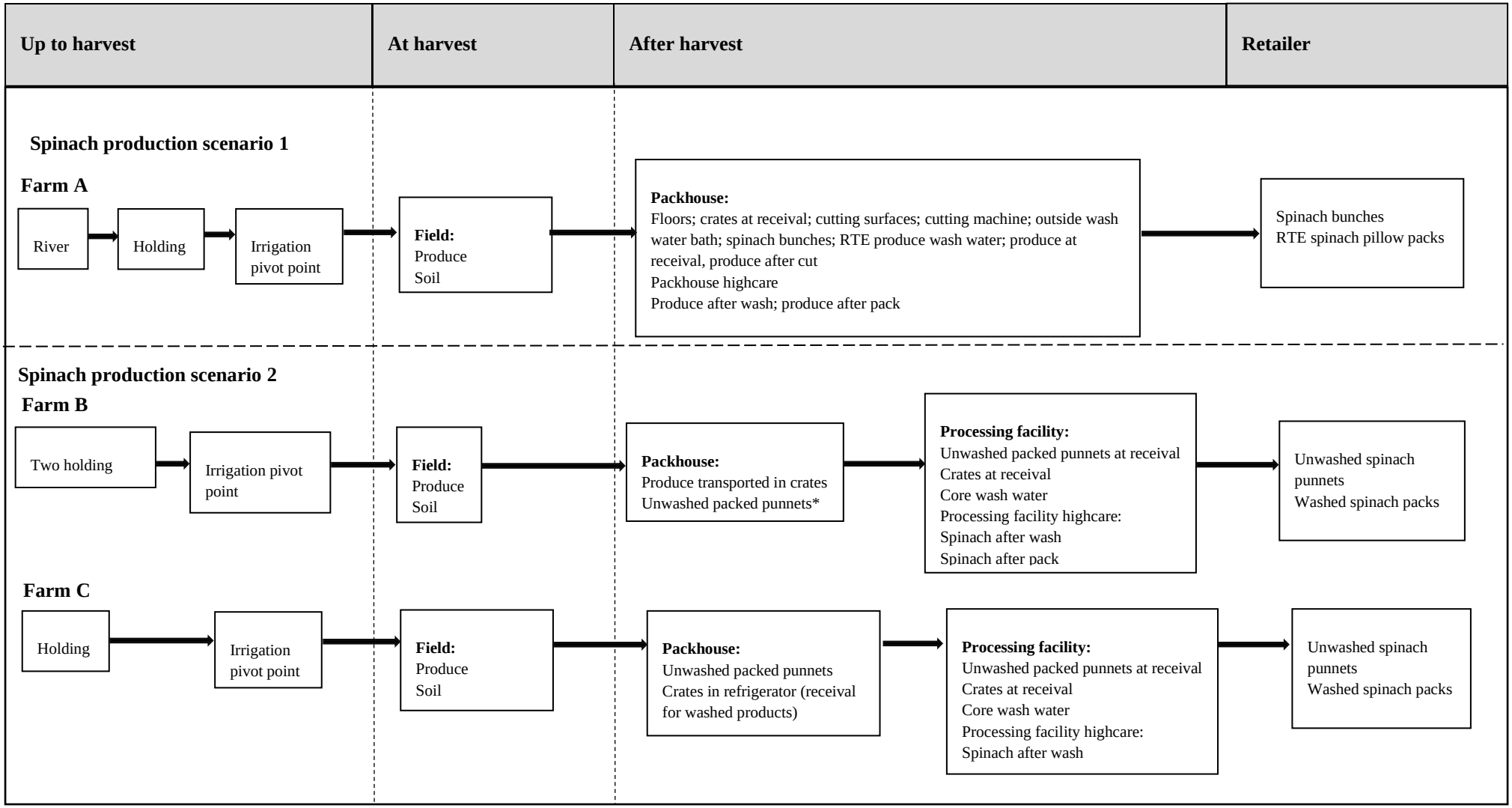

Figure E1: Different sampling points throughout the fresh produce supply chain selected for phenotypic antimicrobial resistance profile and genetic determinant ESBL/AmpC-producing Enterobacteriaceae analysis.
*punnets: plastic containers in which the baby spinach were packaged

Appendix E

Table E1: Matrix-assisted laser desorption time-of-flight identification of extended-spectrum β-lactamase producing Enterobacteriaceae isolated from commercial spinach production systems in Gauteng Province

University of Pretoria Culture number	UPMP code	Published isolate number	Isolate Code	Sample Code	Farm/ Vendor	Trip	Source	Presumptive Organism Identity	Organism best match*		Range**	Consistency category***	Notes	Isolated by
									Score 1	Score 2				
UP_BN_LR_0023	UPMP 588	1	A5	ESBL A2 R1	Farm A	Trip 2	Water reservoir	*Escherichia coli*	2,495	2,46	+++	A	ESBL	Loandi Richter
UP_BN_LR_0024	UPMP 589	2	A6	ESBL A2 R2	Farm A	Trip 2	Water reservoir	*Escherichia coli*	2,47	2,383	+++	A	ESBL	Loandi Richter
UP_BN_LR_0025	UPMP 590	3	A7	ESBL A2 R3	Farm A	Trip 2	Water reservoir	*Escherichia coli*	2,433	2,365	+++	A	ESBL	Loandi Richter
UP_BN_LR_0051	UPMP 616	4	B22	ESBL R1	Farm B	Trip 2	River water	*Escherichia coli*	2,475	2,443	+++	A	ESBL	Loandi Richter
UP_BN_LR_0055	UPMP 620	5	B24	ESBL Pi2,1	Farm B	Trip 2	Irrigation pivot point water	*Escherichia coli*	2,397	2,458	+++	A	ESBL	Loandi Richter
UP_BN_LR_0056	UPMP 621	6	B25	ESBL Pi1,1	Farm B	Trip 2	Irrigation pivot point water	*Escherichia coli*	2,375	2,359	+++	A	ESBL	Loandi Richter
UP_BN_LR_0036	UPMP 601	7	B32	Pi3.1	Farm B	Trip 2	Irrigation pivot point water	*Escherichia coli*	2,388	2,533	+++	A	ESBL	Loandi Richter
UP_BN_LR_0095	UPMP 660	8	B26	Pi1	Farm B	Trip 2	Irrigation pivot point water	*Klebsiella pneumoniae*	2,21	2,4	++	A	ESBL	Loandi Richter
UP_BN_LR_0096	UPMP 2118	9	B27	Pi2	Farm B	Trip 2	Irrigation pivot point water	*Klebsiella pneumoniae*	2,364	2,43	+++	A	ESBL	Loandi Richter
UP_BN_LR_0097	UPMP 662	10	B33	Pi3	Farm B	Trip 2	Irrigation pivot point water	*Klebsiella pneumoniae*	2,435	2,435	+++	A	ESBL	Loandi Richter
UP_BN_LR_0131	UPMP 696	11	B17	WW3	Farm B	Trip 1	Wash water	*Rahnella aquatilis*	1,981	1,984	+	C	ESBL	Loandi Richter
UP_BN_LR_0127	UPMP 692	12	B38	R (composite)	Farm B	Trip 2	River water	*Salmonella spp.*	2,227	2,397	+++	A	ESBL	Loandi Richter
UP_BN_LR_0129	UPMP 694	13	B40	Pi (composite)	Farm B	Trip 2	Irrigation pivot point water	*Salmonella spp.*	2,037	2,271	++	A	ESBL	Loandi Richter
UP_BN_LR_0101	UPMP 2119	14	A12	Pi3	Farm A	Trip 2	Irrigation pivot point water	*Serratia fonticola*	2,346	2,381	+++	A	ESBL	Loandi Richter
UP_BN_LR_0102	UPMP 667	15	A13	Pi1	Farm A	Trip 2	Irrigation pivot point water	*Serratia fonticola*	2,422	2,488	+++	A	ESBL	Loandi Richter
UP_BN_LR_0106	UPMP 2116	16	B15	TR2	Farm B	Trip 1	River water	*Serratia fonticola*	2,234	2,365	++	A	ESBL	Loandi Richter
UP_BN_LR_0135	UPMP 700	17	C2	Sp5URT	Farm C	Trip 1	Unwashed spinach punnet at retailer	*Enterobacter asburiae*	1,873	2,001	++	C	ESBL	Loandi Richter
UP_BN_LR_0045	UPMP 610	18	B23	ESBL Sp3AC	Farm B	Trip 2	Spinach after cut	*Escherichia coli*	2,464	2,407	+++	A	ESBL	Loandi Richter
UP_BN_LR_0047	UPMP 612	19	B34	ESBL Sp3URT	Farm B	Trip 2	Unwashed spinach bunches at retailer	*Escherichia coli*	2,429	2,51	+++	A	ESBL	Loandi Richter
UP_BN_LR_0086	UPMP 651	20	C11	ESBL Sp4RT	Farm C	Trip 2	Spinach at retailer	*Escherichia coli*	2,476	2,552	+++	A	ESBL	Loandi Richter
UP_BN_LR_0089	UPMP 2114	21	B1	TSp1H	Farm B	Trip 1	Spinach at harvest	*Klebsiella pneumoniae*	2,424	2,498	+++	A	ESBL	Loandi Richter

Appendix E

UP_BN_LR_0090	UPMP 655	22	B3	TSp3H	Farm B	Trip 1	Spinach at harvest	*Klebsiella pneumoniae*	2,49	2,523	+++	A	ESBL	Loandi Richter
UP_BN_LR_0091	UPMP 2122	23	B9	Sp1RT	Farm B	Trip 1	Spinach at retailer	*Klebsiella pneumoniae*	2,465	2,522	+++	A	ESBL	Loandi Richter
UP_BN_LR_0092	UPMP 657	24	B10	Sp2RT	Farm B	Trip 1	Spinach at retailer	*Klebsiella pneumoniae*	2,445	2,504	+++	A	ESBL	Loandi Richter
UP_BN_LR_0093	UPMP 658	25	B11	Sp4RT	Farm B	Trip 1	Spinach at retailer	*Klebsiella pneumoniae*	2,512	2,503	+++	A	ESBL	Loandi Richter
UP_BN_LR_0094	UPMP 2121	26	B13	Sp5URT	Farm B	Trip 1	Unwashed spinach bunches at retailer	*Klebsiella pneumoniae*	2,493	2,548	+++	A	ESBL	Loandi Richter
UP_BN_LR_0124	UPMP 689	27	A1	Sp1Re	Farm A	Trip 1	Spinach at receival	*Rahnella aquatilis*	2,055	2,073	++	C	ESBL	Loandi Richter
UP_BN_LR_0125	UPMP 690	28	A2	Sp2Re	Farm A	Trip 1	Spinach at receival	*Rahnella aquatilis*	2,334	2,396	+++	C	ESBL	Loandi Richter
UP_BN_LR_0130	UPMP 695	29	B12	Sp2URT	Farm B	Trip 1	Unwashed spinach bunches at retailer	*Rahnella aquatilis*	1,97	2,044	+	B	ESBL	Loandi Richter
UP_BN_LR_0132	UPMP 697	30	B28	Sp1RT	Farm B	Trip 2	Spinach at retailer	*Rahnella aquatilis*	1,91	1,962	+	C	ESBL	Loandi Richter
UP_BN_LR_0133	UPMP 698	31	B30	Sp2RT2	Farm B	Trip 2	Spinach at retailer	*Rahnella aquatilis*	1,917	1,931	+	B	ESBL	Loandi Richter
UP_BN_LR_0098	UPMP 2123	32	A3	Sp5URT	Farm A	Trip 2	Unwashed spinach punnet at retailer	*Serratia fonticola*	2,074	2,42	+++	A	ESBL	Loandi Richter
UP_BN_LR_0099	UPMP 664	33	A10	Sp1D	Farm A	Trip 2	Spinach at dispatch	*Serratia fonticola*	2,267	2,399	+++	A	ESBL	Loandi Richter
UP_BN_LR_0100	UPMP 665	34	A11	Sp3URT	Farm A	Trip 2	Unwashed spinach punnet at retailer	*Serratia fonticola*	2,191	2,294	++	A	ESBL	Loandi Richter
UP_BN_LR_0104	UPMP 2124	35	B4	TSp2Re	Farm B	Trip 1	Spinach at receival	*Serratia fonticola*	2,225	2,245	++	A	ESBL	Loandi Richter
UP_BN_LR_0105	UPMP 2125	36	B8	TSp2AP	Farm B	Trip 1	Spinach after pack	*Serratia fonticola*	2,347	2,344	+++	C	ESBL	Loandi Richter
UP_BN_LR_0107	UPMP 672	37	B36	Sp5RT	Farm B	Trip 2	Spinach at retailer	*Serratia fonticola*	2,416	2,411	+++	A	ESBL	Loandi Richter
UP_BN_LR_0108	UPMP 673	38	C1	Sp4URT	Farm C	Trip 1	Unwashed spinach punnet at retailer	*Serratia fonticola*	2,189	2,255	++	A	ESBL	Loandi Richter
UP_BN_LR_0109	UPMP 2126	39	C4	Sp2Re	Farm C	Trip 1	Spinach at receival	*Serratia fonticola*	2,336	2,418	+++	A	ESBL	Loandi Richter
UP_BN_LR_0111	UPMP 676	40	C6	Sp5URT	Farm C	Trip 2	Unwashed spinach punnet at retailer	*Serratia fonticola*	2,423	2,378	+++	A	ESBL	Loandi Richter
UP_BN_LR_0112	UPMP 2127	41	C7	Sp4URT	Farm C	Trip 2	Unwashed spinach punnet at retailer	*Serratia fonticola*	2,406	2,431	+++	A	ESBL	Loandi Richter
UP_BN_LR_0113	UPMP 678	42	C8	Sp3URT	Farm C	Trip 2	Unwashed spinach punnet at retailer	*Serratia fonticola*	2,336	2,357	+++	A	ESBL	Loandi Richter
UP_BN_LR_0114	UPMP 2128	43	C9	Sp2URT	Farm C	Trip 2	Unwashed spinach punnet at retailer	*Serratia fonticola*	2,305	2,342	+++	A	ESBL	Loandi Richter

Appendix E

UP_BN_LR_0116	UPMP 681	44	C12	Sp2RT	Farm C	Trip 2	Spinach at retailer	*Serratia fonticola*	2,382	2,364	+++	A	ESBL	Loandi Richter
UP_BN_LR_0117	UPMP 682	45	C13	JJSp2U	Farm C	Trip 2	Unwashed spinach punnet at packhouse	*Serratia fonticola*	2,241	2,202	++	A	ESBL	Loandi Richter
UP_BN_LR_0118	UPMP 683	46	C14	JJSp3U	Farm C	Trip 2	Unwashed spinach punnet at packhouse	*Serratia fonticola*	2,338	2,425	+++	A	ESBL	Loandi Richter
UP_BN_LR_0120	UPMP 685	47	C16	JJSp2Re	Farm C	Trip 2	Spinach at receival (packhouse)	*Serratia fonticola*	2,343	2,299	++	A	ESBL	Loandi Richter
UP_BN_LR_0123	UPMP 2129	48	C26	Sp2Re	Farm C	Trip 2	Spinach at receival	*Serratia fonticola*	2,476	2,347	+++	A	ESBL	Loandi Richter
UP_BN_LR_0110	UPMP 675	49	C5	H2	Farm C	Trip 1	Contact surfaces (packhouse)	*Serratia fonticola*	2,32	2,387	+++	A	ESBL	Loandi Richter
UP_BN_LR_0121	UPMP 686	50	C17	JJH2	Farm C	Trip 2	Contact surfaces (packhouse)	*Serratia fonticola*	2,338	2,375	+++	C	ESBL	Loandi Richter
UP_BN_LR_0087	UPMP 652	51	C18	ESBL JJSp2U	Farm C	Trip 2	Unwashed spinach at dispatch (packhouse)	*Escherichia coli*	2,525	2,523	+++	A	ESBL	Loandi Richter
UP_BN_LR_0088	UPMP 653	52	C20	ESBL D1	Farm C	Trip 2	Holding dam water (source water)	*Escherichia coli*	2,446	2,63	+++	A	ESBL	Loandi Richter
UP_BN_LR_0134	UPMP 699	53	C3	JJCS2	Farm C	Trip 1	Contact surfaces (packhouse)	*Rahnella aquatilis*	2,405	2,436	+++	C	ESBL	Loandi Richter
UP_BN_LR_0126	UPMP 2115	54	B37	R3	Farm B	Trip 2	River water	*Salmonella spp.*	2,302	2,282	++	A	ESBL	Loandi Richter
UP_BN_LR_0128	UPMP 693	55	B39	Pi3	Farm B	Trip 2	Irrigation pivot point water	*Salmonella spp.*	2,19	2,147	++	C	ESBL	Loandi Richter
UP_BN_LR_0103	UPMP 668	56	B2	TSp2H	Farm B	Trip 1	Spinach at harvest	*Serratia fonticola*	2,06	2,206	++	A	ESBL	Loandi Richter
UP_BN_LR_0115	UPMP 2131	57	C10	Sp1URT	Farm C	Trip 2	Unwashed spinach punnet at retailer	*Serratia fonticola*	2,205	2,349	++	A	ESBL	Loandi Richter
UP_BN_LR_0119	UPMP 684	58	C15	JJSp3Re	Farm C	Trip 2	Spinach at receival (packhouse)	*Serratia fonticola*	2,155	2,292	++	A	ESBL	Loandi Richter
UP_BN_LR_0122	UPMP 687	59	C19	JJCS2	Farm C	Trip 2	Contact surfaces (packhouse)	*Serratia fonticola*	2,194	2,314	++	A	ESBL	Loandi Richter

Control strain: Bruker Bacterial Test Standard (BTS): consists of a manufactured extract of *Escherichia coli* DH5 alpha. Isolates 1 – 48 were subsequently confirmed as ESBL/AmpC-producing Enterobacteriaceae. * All isolates were measured in duplicate; ** Range description: 2.300- 3.000 (+++) highly probable species identification; 2.000- 2.299 (++) secure genus identification, probable species identification; 1.700- 1.999 (+) probable genus identification; 0.000- 1.699 (-) not reliable identification; *** Consistency categories description: (A) Species consistency; (B) Genus consistency; (C) No consistency, consider synonyms of names

Appendix F

Table F1: Assembly metrics of Enterobacteriales subjected to whole genome sequencing analysis

Strain	Organism Identity	Assembly metrics									
		Total_reads	Total_yield	Clean_reads	bases	CoverageDepth	Contig_num	GC_content	N50_value	Longest_contig	Total_bases
UPMP2117	*Escherichia coli*	4656884	681551924	4635628	677534287	79	3453	53.59	8457	77645	9119260
UPMP2120	*Escherichia coli*	4628474	677073919	4608558	673548562	134	59	50.86	263026	476299	4718037
UPMP2130	*Escherichia coli*	6606040	965271619	6578754	960087904	96	749	54.14	31911	141185	10191997
UPMP2112	*Klebsiella pneumoniae*	5619120	820747033	5588856	814850638	153	64	57.26	325454	834005	5502104
UPMP2118	*Klebsiella pneumoniae*	5333708	780086995	5311000	774790087	152	50	57.39	361868	759884	5347823
UPMP2121	*Klebsiella pneumoniae*	6352056	930649424	6323756	924149035	179	42	57.21	481688	974208	5452642
UPMP2122	*Klebsiella pneumoniae*	5311234	778978847	5289648	773609931	150	45	57.21	293800	974089	5453105
UPMP2116	*Serratia fonticola*	5865526	856345563	5844598	851905324	70	70	53.65	285230	767470	5541108
UPMP2119	*Serratia fonticola*	6741632	987774568	6719014	981850580	158	53	53.77	283491	663195	5629233
UPMP2123	*Serratia fonticola*	6534130	956489997	6507614	951520153	168	48	53.84	406312	684839	5659091
UPMP2124	*Serratia fonticola*	6479250	947810080	6451796	942715441	90	102	55.50	235064	767529	10825220
UPMP2125	*Serratia fonticola*	6598416	966038175	6571032	960267671	85	137	55.45	227481	1208867	11118130
UPMP2126	*Serratia fonticola*	5777244	846484833	5755366	841758099	53	4619	53.64	14607	511186	15473115
UPMP2127	*Serratia fonticola*	6725308	984845445	6698976	979365429	83	342	55.27	131504	392216	11619906
UPMP2128	*Serratia fonticola*	5839876	855362335	5816670	850890182	140	119	53.60	182838	377140	6122714
UPMP2129	*Serratia fonticola*	5272916	770986391	5249834	766762201	131	108	53.61	239988	658724	6168315
UPMP2131	*Serratia fonticola*	5709994	835397091	5686374	830609084	54	501	54.14	135948	499037	15805578

Appendix F

Table F2: Virulence factors associated with *Salmonella* pathogenicity islands in the *Salmonella enterica* strain isolated from river water following whole genome sequence analysis

Salmonella Pathogenicity Island	Virulence factors																	
C63PI	cheD	vexA	rtxA	rtxA	exeE	exeE	fyuA	cpsB	isdD	ratB	phzE1	xcpU	lbpA	lpg1449	icmO/dotL	fyuA	flgK	cwp84	bscC
CS54_island	adeG	mtrD	bscS	sopB/sigD	lpg2160	acrB	mrkC	fliN	nueB	flmD	basG	yapC	cheA	flgL	cylB	adsA	sseJ	tviE	sca2
	pchH	pchI	fliF	lbpB	espR	lem17	lem16	licD	faeH	spaF	wcbR	boaB	boaA	lef	virB10	rtxA	rtxA	lfiQ	
Not_named	ssaD	cdsR	irp1	esaA	rvhB6a	cegC2	fliS	irp1	tssA	virA	ipaD	tagF/pppB	ppkA	p97	lpg3000	ravY	sdeC/laiC	sdeB	sidE/laiD
	vipA	tbpB	pilO	faeG	ecbA	coxH4	pseB	kpsC	flhF	cesC	cer	BC0552							
SPI-1	entD	pscD	rcsB	hsiE1	fliR	rrgC	pvdH	pchH	fhaB	psaE	rtxA	ssaC	dotU1	popN	xcpW	mhp271	mbtA	icmF/tssM	vipB/tssC
	CBUD_1884	tssF-5	virB4	tapT															
SPI-2	ssaV	ssaC	sseC	ssaN	ssaD	sseF	ssaJ	sseG	ssaK	ssaL	sseB	sseD	sscA	sscB	spiC/ssaB	sseE	ssaM	ssaO	sseA
	ssaP	ssaE	ssaI	ssaH	ssaG	yscV/lcrD	vcrD	escV	vscN	copB	yscN	pscN	invC/sctN	gspE	sseI/srfH	legG2	cetCb2	nagH	virB11
	virB6-4	cnf	scpB	scpA	pscC	lbpA	lpg1924	mavB	lpg1147	legK2	icmB/dotO	flgA	cagA	HI0867	iglI1	manC	chuA	jlpA	fliM
	lpxB	virB4	pomB																
SPI-3	afaC-VII	psaC	afaC-VIII	afaC-III	afaC-I	draC	coxU1	rhs/PAAR	rvhB6c	esp	fimB	sinH	ssaL	fliI	fliH	exoS	pilU	hpuA	lpg1959
	clbN	ansP	csaA	cfaA	papJ	aafD	flgL	rtxE	lfiE	ascV									
SPI-4	lem21	iutA	iutA	vWbp	algX	espK	mbtF	mavN	fepD	cylB	rtxB	flhA	cylF	shuS	flgN	fha1	lgt3/legC5	fimD	motA
	yhxB/manB	chuW	chuS	hlyA	EF0818	prgB/asc10	cpeE	dotD	CD2830	nagL	neuC1	wcbF	wcbR	bspI3	boaB	bscQ	trwJ2	cytK	virB6-2
	bauC	bauE	tapT	galU															
SPI-5	sopB/sigD	pipB	prsA2	ravH	cetCb2	nagI	vpadF	flgI	pilG	icmX	wbbM	cagA	waaQ	wbkA	inhA	flgE	flgE	barB	manB
	flgK	scpB	scpA	ipgD	ipaC	sigA	sipB/sspB	sopE2	oatA	lpg2552	lpg2239	lpg1959	pieD/lirF	legL3	wipB	fepD	bsc1	iglF1	neuB
	EF0149	coxCC6	CBU_0635	icmE	spaC	maf4	pseD/maf2	Cj1438c	Cj1422c	Cj1421c	lfhA	flgN	tapN	tapN	aopN				
SPI-9	icmF1/tssM1	vgrG/tssI	vgrG1b	vgrG1a	pvdL	gadC	mtrC	clbB	slpA	C2I	vopC	vscS2	rhs/PAAR	sinH	shdA	motD	fliH	pcr2	phoP
	flhF	cyaE	rtxE	fleQ/flrC	fliA	fliA	mshN												
SPI-13	flhF	(algL	iroE	flrA	fleQ/flrC	flpH	exlB	phzE1	motB	xcpS	sidJ	allS	flgH	wcbQ	yscV/lcrD	irp1	acfD	iucC	ratB
	ssaK	clpV1	hsiG1	fha1	ppkA	pchI	chpA	plcH	algJ	inlK	iucC	irp1	mrkB	sfaA	iucC	ironN	sat	CBU_0372	toxA
	CT622	flgA	tagAB-5	boaA	brkA	fhaB	aipA	flmD	cheW	cheW	fliI	motX	mshE	flpL	flpL	tapD	bauD		
SPI-14	lem21	vWbp	cylB	cylF	lgt3/legC5	EF0818	cpeE	CD2830	nagL	trwJ2	cytK	yopM	vopL	srtC1	cap8D	can	shuU	rvhB6e	prsA2
	lirA	rtxA	sidG	sdeA/laiA	pflA	lgtC	iglJ1	flmK	ompA	chuU	toxB	CBUD_2154	virB10	BAS2037	bfmS				

Appendix F

Table F3: Virulence factors detected using whole genome sequencing in *Escherichia coli* from water and spinach samples

Accession	Strain	ST	Serotype	Contig	Virulence Genes																					
					lpfA[1]	*gad*[2]	*terC*[3]	*astA*[4]	*fyuA*[5]	*irp2*[6]	*iss*[7]	*sitA*[8]	*traT*[9]	*chuA*[10]	*cia*[11]	*hlyF*[12]	*hra*[13]	*iroN*[14]	*iucC*[15]	*iutA*[16]	*mchB*[17]	*mchC*[18]	*mchF*[19]	*ompT*[20]	*papC*[21]	*vat*[22]
SAMN15421728	UPMP2120	ST58	O75:H9	JACNYS010000001.1		+																				
				JACNYS010000006.1		+																				
				JACNYS010000011.1	+																					
				JACNYS010000004.1			+																			
				JACNYS010000014.1			+																			
SAMN15421725	UPMP2117	ST117	O11:H4	JACNYT010000061.1										+												
				JACNYT010000290.1											+											
				JACNYT010000065.1					+																	
				JACNYT010000267.1												+										
				JACNYT010000044.1													+									
				JACNYT010000118.1														+								
				JACNYT010000109.1						+																
				JACNYT010000106.1							+															
				JACNYT010000118.1							+															
				JACNYT010000115.1															+							
				JACNYT010000115.1																+						
				JACNYT010000031.1	+																					
				JACNYT010000032.1																	+					
				JACNYT010000032.1																		+				
				JACNYT010000032.1																			+			
				JACNYT010000267.1																				+		
				JACNYT010000036.1																				+		
				JACNYT010000084.1																					+	
				JACNYT010000115.1								+														
				JACNYT010000018.1			+																			
				JACNYT010000271.1			+																			
				JACNYT010000025.1									+													
				JACNYT010000262.1																						+
SAMN15421738	UPMP2130	ST10	O8:H17	JACNYN010000111.1				+																		
				JACNYN010000160.1					+																	
				JACNYN010000165.1						+																
				JACNYN010000053.1							+															
				JACNYN010000039.1								+														
				JACNYN010000004.1			+																			
				JACNYN010000090.1			+																			
				JACNYN010000142.1									+													
				JACNYN010000143.1									+													
				JACNYN010000034.1									+													

1 Long polar fimbriae
2 Glutamate decarboxylase
3 Tellurium ion resistance protein
4 EAST-1 heat-stable toxin
5 Siderophore receptor
6 High molecular weight protein 2 non-ribosomal peptide synthetase
7 Increased serum survival
8 Iron transport protein
9 Outer membrane protein complement resistance
10 Outer membrane hemin receptor
11 Colicin ia
12 Hemolysin F
13 Heat-resistant agglutinin
14 Enterobactin siderophore receptor protein
15 Aerobactin synthetase
16 Ferric aerobactin receptor
17 Microcin H47 part of colicin H
18 MchC protein
19 ABC transporter protein MchF
20 Outer membrane protease (protein protease 7)
21 Outer membrane usher P fimbriae
22 Vacuolating autotransporter toxin

Appendix F

Table F4: Virulence factors detected using whole genome sequencing in *Klebsiella pneumoniae* from water and spinach samples

Accession	Strain	ST	Serotype	Contig	Virulence Genes																					
					kfuA[1]	kfuB[2]	kfuC[3]	mrkA[4]	mrkB[5]	mrkC[6]	mrkD[7]	mrkF[8]	mrkH[9]	mrkI[10]	mrkJ[11]	fyuA[12]	irp1[13]	irp2[14]	ybtA[15]	ybtE[16]	ybtP[17]	ybtQ[18]	ybtS[19]	ybtT[20]	ybtU[21]	ybtX[22]
SAMN15375861	UPMP2112	ST3559	KL27	O4	JACAAL010000002.1				+																	
					JACAAL010000002.1					+																	
					JACAAL010000002.1						+															
					JACAAL010000002.1							+														
					JACAAL010000002.1								+													
					JACAAL010000002.1									+												
					JACAAL010000002.1										+											
					JACAAL010000002.1											+										
SAMN15421726	UPMP 2118	ST15	KL24	O1v1	JACBJB010000003.1	+																				
					JACBJB010000003.1		+																			
					JACBJB010000003.1			+																		
					JACBJB010000009.1				+																	
					JACBJB010000009.1					+																
					JACBJB010000009.1						+															
					JACBJB010000009.1																					
					JACBJB010000009.1							+														
					JACBJB010000009.1								+													
					JACBJB010000009.1									+												
					JACBJB010000009.1										+											
					JACBJB010000009.1											+										
SAMN15421722	UPMP2114	ST985	KL39	O1v2	JACBJE010000001.1											+										
					JACBJE010000001.1												+									
					JACBJE010000001.1													+								
					JACBJE010000004.1				+																	
					JACBJE010000004.1					+																
					JACBJE010000004.1						+															
					JACBJE010000004.1							+														
					JACBJE010000004.1								+													
					JACBJE010000004.1									+												
					JACBJE010000004.1										+											
					JACBJE010000001.1														+							

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					JACBJE010000001.1
					JACBJE010000001.1
					JACBJE010000001.1
					JACBJE010000001.1
					JACBJE010000001.1
					JACBJE010000001.1
					JACBJE010000001.1
SAMN15421729	UPMP2121	ST985	KL39	O1v2	JACBIZ010000001.1
					JACBIZ010000001.1
					JACBIZ010000001.1
					JACBIZ010000011.1
					JACBIZ010000011.1
					JACBIZ010000011.1
					JACBIZ010000011.1
					JACBIZ010000011.1
					JACBIZ010000011.1
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					JACBIZ010000001.1
					JACBIZ010000001.1
					JACBIZ010000001.1
					JACBIZ010000001.1
SAMN15421730	UPMP2122	ST985	KL39	O1v1	JACBIY010000001.1
					JACBIY010000001.1
					JACBIY010000001.1
					JACBIY010000009.1
					JACBIY010000009.1
					JACBIY010000009.1
					JACBIY010000009.1
					JACBIY010000009.1
					JACBIY010000009.1
					JACBIY010000009.1
					JACBIY010000009.1

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JACBIY010000001.1														+						
JACBIY010000001.1															+					
JACBIY010000001.1																+				
JACBIY010000001.1																	+			
JACBIY010000001.1																		+		
JACBIY010000001.1																			+	
JACBIY010000001.1																				+
JACBIY010000001.1																				

1 Klebsiella Ferric ionic-uptake system A

2 Klebsiella Ferric ionic-uptake system B

3 Klebsiella Ferric ionic-uptake system C

4 Type 3 fimbriae major subunit protein

5 Type 3 fimbriae chaperone

7 Type 3 fimbriae adhesin

8 Type 3 fimbriae minor subunit protein

9 Regulatory protein

10 LuxR-type transcriptional regulator

11 Phosphodiesterase

12–Siderophore receptor

13 Yersinibactin biosynthesis protein

14 Yersiniabactin biosynthesis protein

15 Transcriptional regulator

16 Siderophore

17 Yersiniabactin transport protein

18 Putative ABC transporter protein

19 Siderophore

Table F5: Virulence factors detected in multiple *Serratia fonticola* contigs from water and spinach samples using whole genome sequencing

Virulence factors	Strain	UPMP2123	UPMP2124	UPMP2125	UPMP2126	UPMP2127	UPMP2128	UPMP2129	UPMP2131	UPMP2119	UPMP2116
	Contig	JACBIX01000000X	JACNYR01000000X	JACNYQ01000000X	JACNYP01000000X	JACNYO01000000X	JACBIW01000000X	JACBIV01000000X	JACNYM01000000X	JACBJA01000000X	JACBJC01000000X
	fimE		15,1								15,1
	fimB		15,1								15,1
	fimG									9,1	
	fimC									9,1	
	fimD	1,1		8,1	10,1	7,1	5,1	6,1	5,1	9,1/12,1	3,1
	lpxC	1,1		8,1	10,1	7,1	5,1	6,1	5,1	6,1	3,1
	htpB	1,1	15,1	19,1		54,1	49,1	43,1	34,1	10,1	15,1
	pilW	2,1		1,1					25,1	3,1	
	yagZ/ecpA	2,1		24,1	13,1	12,1	9,1	7,1	15,1	26,1	5,1/9,1
	yagW/ecpD	2,1		24,1	13,1	12,1	9,1	7,1	15,1	26,1	9,1
	galU	2,1		14,1	1,1	10,1	13,1	13,1	15,1	14,1	11,1
	wbaP/rfbP	2,1									11,1
	manB	2,1			1,1				15,1		
	msbA	2,1		21,1	1,1	17,1	16,1	15,1	6,1	3,1	11,1
	rffG										11,1
	ompA	2,1		21,1	1,1	17,1	16,1	15,1	6,1	3,1	2,1
	hcp-2	2,1		7,1	1,1	35,1	11,1	12,1	6,1	3,1	2,1
	vipB/mglB	2,1		7,1	1,1	35,1	11,1	12,1	6,1	3,1	2,1
	fepA	3,1		2,1		1,1	26,1	2,1	13,1	2,1	6,1
	fepC	3,1		2,1		1,1	26,1	2,1	13,1	2,1	6,1
	fepG	3,1		2,1		1,1	26,1	2,1	13,1	2,1	6,1
	entS	3,1		2,1		1,1	26,1	2,1	13,1	2,1	6,1
	fepB	3,1		2,1		1,1	26,1	2,1	13,1	2,1	6,1
	entA			2,1		1,1		2,1	13,1	2,1	6,1
	entE	3,1		2,1		1,1	26,1	2,1	13,1	2,1	6,1
	entB	3,1		2,1		1,1	26,1	2,1	13,1	2,1	6,1
	katA	3,1			4,1			2,1		2,1	5,1
	shuA	4,1		18,1	11,1	6,1	7,1	1,1	2,1	1,1	2,1
	shuS	4,1				6,1	7,1	1,1	2,1	1,1	
	kdsA	5,1	13,1	17,1	8,1				7,1	1,1	13,1
	chuS				11,1				7,1		2,1
	chuW	5,1		24,1	8,1	37,1	34,1		7,1	1,1	
	chuY	5,1	13,1	24,1	8,1	37,1	34,1			1,1	13,1
	iroN	6,1							1,1	7,1	
	IlpA	7,1		5,1		19,1	18,1	16,1	1,1	3,1	1,1
	lpxA	7,1		5,1		19,1	18,1	16,1	1,1	3,1	1,1
	clpP	9,1		20,1	5,1	4,1	4,1	5,1	23,1	3,1	4,1
	gtrB	10,1		2,1	7,1	3,1	3,1	4,1	4,1	2,1	6,1
	mgtB	11,1	12,1	11,1		13,1	15,1	9,1	8,1	13,1	12,1
	flhD	11,1		11,1		13,1	15,1	9,1	8,1	13,1	12,1
	flhC	11,1		11,1		13,1	15,1	9,1	8,1	13,1	12,1
	motA	11,1		11,1		13,1	15,1	9,1	8,1	13,1	12,1
	cheW	11,1	12,1	11,1		13,1	15,1	9,1	8,1	13,1	12,1
	cheD	11,1	12,1	11,1		13,1	15,1	9,1	8,1	13,1	12,1
	cheR	11,1	12,1	11,1		13,1	15,1	9,1	8,1	13,1	12,1
	cheB	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1

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Virulence factors

cheY	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
cheZ	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
flhB	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
flhA	11,1		11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
pilW	11,1				13,1	15,1	9,1	8,1	13,1	2,1/12,1
flgB	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
flgC	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
flgD	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
flgE	11,1		11,1	18,1				8,1		12,1
flgF	11,1	12,1	11,1	18,1		15,1	9,1	8,1	13,1	12,1
flgL			11,1	18,1		15,1	9,1	8,1		12,1
flgG	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
flgH	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
flgI	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
flgJ	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
flgK	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
fliR	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
fliQ	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
fliP	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
fliN	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
fliM	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
fliJ	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
fliI	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
fliG	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
fliF	11,1	12,1	11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
fliE	11,1		11,1	18,1	13,1	15,1	9,1	8,1	13,1	12,1
fliS	11,1		11,1	18,1	13,1	15,1	9,1	8,1		
fliA	11,1		11,1	18,1	13,1	15,1	9,1	8,1		
fliZ	11,1		11,1	18,1	13,1	15,1	9,1	8,1		
rfaD	12,1	17,1	6,1	14,1	11,1	14,1	14,1	14,1	11,1	17,1
iucB	15,1	20,1	30,1		21,1	19,1	19,1	22,1	17,1	20,1
iucC	15,1	20,1			21,1	19,1	19,1	22,1	1,1	20,1
iutA	15,1	20,1			21,1	19,1	19,1	22,1		20,1
iucD		20,1						22,1		20,1
luxS	23,1	24,1	26,1		54,1	23,1	22,1		12,1	24,1
papD			5,1	3,1					3,1	1,1
katA			31,1			6,1		19,1		
kdsA					61,1	44,1	40,1			
chuW							31,1			
chuY							31,1			
sodB								2,1		
icl								16,1		
iroB									24,1	