

**THE PREVALENCE AND CHARACTERISATION OF *ESCHERICHIA COLI*
ON FRESH PRODUCE FROM SELECTED FARMS, RETAIL OUTLETS
AND MARKETS IN THE WESTERN CAPE**

By

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DECLARATION

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ABSTRACT

South Africa is a water scarce country and farmers are forced to irrigate crops with river water. Contamination of South African rivers has been reported and the carry-over of bacteria from river water to produce has been confirmed. Foodborne outbreaks linked to fresh produce are increasing world-wide.

A total of 151 fresh produce samples (lettuce, tomatoes, beans, peas, coriander, basil, mint, rocket, thyme, spinach, cabbage, parsley and sprouts) were sourced from small-scale and commercial farms, farmers' markets and retail outlets. Total coliforms (TC) and *E. coli* loads on the produce were determined with Colilert-18. Isolates were phenotypically characterised and identified with the API system and the *E. coli* identification confirmed with *uidA* PCR. Sixty-three *E. coli* isolates were identified. Three were not identified as *E. coli* with the API system but were positive for the *uidA* gene.

The TC loads for the produce from the farms, farmers' markets and retail outlets were all in the range of log 3 to log 8.38 MPN.100 mL⁻¹. *Escherichia coli* was found to be most prevalent on produce samples from farmers' markets with the highest *E. coli* load (log 7.38 MPN.100 mL⁻¹) on cabbage sampled from a commercial farm. *Escherichia coli* were present on 8% of the produce samples. The maximum TC and *E. coli* loads found on the fresh produce were log 8.38 and log 7.38 MPN.100 mL⁻¹, respectively. The lowest risk in terms of TC and *E. coli* presence and load was observed on fresh produce from retail outlets and the highest risk was on fresh produce from farmers' markets.

Phenotypic dendrograms and a PCA plot were statistically constructed to determine similarity groupings of the isolates and three main *E. coli* clusters were formed. These three clusters could not be directly linked to a specific produce type or source type. A larger variation *E. coli* phenotypes was observed present on fresh produce within the three clusters.

All *E. coli* isolates were also subjected to triplex and multiplex PCR analysis to identify their phylogenetic groups and the presence of INPEC and ExPEC strains. Fourteen isolates belonged to genotypic group A₀, 11 to A₁, 20 to B₁, 7 to B₂₃ and 11 to D₂. Thus a large variation *E. coli* genotypes are present but it cannot be linked to a specific source type or produce type. Multiplex PCR testing for INPEC revealed that none of the *E. coli* isolates were carriers of the INPEC genes. The isolates were also tested for the presence of ExPEC gene sequences: *papA*, *papC*, *sfa/foc*, *iutA*, *kpsMT II* and *afa/dra*. None of the isolates were classified as ExPEC (which required the presence of two or more genes) but three of the isolates did test positive for the presence of the *kpsMT II* gene. The latter could indicate that potentially pathogenic *E. coli* can be evolving in the environment and increase the risk of pathogenic *E. coli* occurring on fresh produce.

In conclusion, the presence of *E. coli* (commensal or pathogenic) on fresh produce is unacceptable according the South African Department of Health. According to this study the identification of *E. coli* types could not be correlated with the presence of *E. coli* on the different

produce types and thus the presence of *E. coli* on fresh produce is unpredictable. It is recommended that extensive safety precautions should be in place throughout every step in the production chain from harvest to the consumer's kitchen to reduce the probability of contamination of fresh produce.

UITTREKSEL

Suid-Afrika is 'n waterskaars land en boere word gedwing om rivier water te gebruik vir gewas besproeiing. Kontaminasie van Suid-Afrikaanse riviere is al telkemale aangemeld en die oordrag van bakterieë vanaf rivierwater na vars produkte is al voorheen bevestig. Voedselverwante uitbrake wat gekoppel is aan vars produkte is besig om wêreldwyd toe te neem.

'n Totaal van 151 vars produk monsters (blaarslaai, tamaties, boontjies, ertjies, koljander, basilie, kruisement, roket, tiemie, spinasie, kool, pietersielie en spruite) was verkry van klein-skaalse en kommersiële plase, plaasmarkte en kettingwinkels. Totale kolivorme (TK) en *E. coli* tellings op die vars produkte is bepaal deur middel van Colilert-18. Isolate word fenotipies gekarakteriseer en geïdentifiseer met die API sisteem en die *E. coli* identifikasie is bevestig met *uidA* PKR. Drie-en-sestig *E. coli* isolate is geïdentifiseer. Drie is nie met met die API sisteem as *E. coli* geklassifiseer nie, maar was wel positief vir die *uidA* geen.

Die TK tellings vir die vars produkte van die plase, plaasmarkte en kettingwinkels was almal in die reeks van log 3 tot log 8.38 MPN.100 mL⁻¹. *Escherichia coli* teenwoordigheid was die meeste op groente monsters van plaasmarkte, maar die hoogste *E. coli* telling (log 7.83 MPN.100 mL⁻¹) was op 'n kool monster van 'n kommersiële plaas. *Escherichia coli* was teenwoordig op 8% van die vars produk monsters. Die maksimum TK en *E. coli* wat teenwoordig was op die vars produkte was log 8.38 en log 7.38 MPN.100 mL⁻¹ onderskeidelik. Die laagste risiko in terme van TK en *E. coli* teenwoordigheid en tellings is waargeneem op vars produkte van kettingwinkels en die hoogste risiko is op vars produkte van plaasmarkte.

Fenotipiese dendrogramme en 'n PKA plot is statisties gekonstrueer om ooreenstemende groepe van isolate te identifiseer en drie hoof groepe is gevorm. Daar kon geen direkte verband gevind word tussen hierdie drie groepe en 'n spesifieke produk-tipe of 'n spesifieke bron-tipe nie. 'n Groter variasie in *E. coli* fenotipes teenwoordig op die vars produkte is waargeneem binne die drie groepe.

Alle *E. coli* isolate was onderworpe aan tripleks en multipleks PKR analise om die filogenetiese groep van elke isolaat te bepaal en of enige INPEC of ExPEC stamme teenwoordig is. Veertien isolate behoort aan genotipiese groep A₀, 11 aan A₁, 20 aan B₁, 7 aan B₂ en 11 aan D₂. Dus is 'n groot variasie *E. coli* genotipes teenwoordig maar dit kan nie gekoppel word aan 'n spesifieke produk-tipe of bron-tipe nie. Multipleks PKR analise vir INPEC het gewys dat geeneen van die *E. coli* isolate enige INPEC gene dra nie. Die isolate is ook getoets vir die teenwoordigheid van ExPEC geen volgordes: *papA*, *papC*, *sfa/foc*, *iutA*, *kpsMT II* en *afa/dra*. Geeneen van die isolate is geklassifiseer as ExPEC (wat die teenwoordigheid van twee of meer gene vereis) nie, maar drie van die isolate het wel positief getoets vir die teenwoordigheid van die *kpsMT II* geen. Laasgenoemde kan 'n aanduiding wees dat potensiële patogeniese *E. coli* in die omgewing kan ontwikkel en dus dan die risiko van die teenwoordigheid van patogeniese *E. coli* op vars produkte sal verhoog.

Ter afsluiting, die teenwoordigheid van *E. coli* (nie-patogenies en patogenies) op vars produkte is onaanvaarbaar volgens die Suid-Afrikaanse Departement van Gesondheid. Volgens hierdie studie kan die identifisering van *E. coli* tipes nie gekorreleer word met die teenwoordigheid van *E. coli* op verskillende produk-tipes nie en dus is die teenwoordigheid van *E. coli* op vars produkte onvoorspelbaar. Dit word aanbeveel dat ekstensiewe voorsorgmaatreëls in plek moet wees in elke stap dwarsdeur die produksie ketting, vanaf oestyd tot in die verbruiker se kombuis, om die moontlikheid van vars produk kontaminasie te verminder.

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

CHAPTER 1

INTRODUCTION

Health is a constant topic on the radar. Every consumer wants to look, feel and be healthy. The basis of a healthy diet consists of fresh fruit and vegetables as these are generally conceived as healthy, unprocessed, relatively cheap and available. Consumers are thus prone to purchase these products for basic food preparation. Fresh produce is considered a healthy food product but whether it is microbiologically safe, is however, another question (Garret *et al.*, 2003).

There is increasing evidence that the consumption of contaminated fresh produce is a major factor contributing to foodborne diseases (Lynch *et al.*, 2009). Besides the negative health aspects for the consumer, this can be damaging to the food industry as it will result in loss of sales. If outbreaks increase and consumers' consumption decreases as result, a country's economy can be negatively impacted. Thus, it is essential to invest in the microbiological surveillance of food products so as to subsequently ensure consumer safety (Heaton & Jones, 2007).

Water scarcity is a reality for South Africa (SA) and this has an impact both on the economy (Turton, 2008) and more directly on the agricultural sector as farmers need water to produce food. Irrigation water sources can either be municipal water, stored rain water, ground water accessed through boreholes or river water. The lack of rain contributes directly to water scarcity. Many SA rivers have been reported to be unsuitable for irrigation purposes as a result of the high levels of faecal and microbial contamination (Bezuidenhout *et al.*, 2002; Obi *et al.*, 2002; Lin *et al.*, 2003; Barnes & Taylor, 2004; Paulse *et al.*, 2007; Britz *et al.*, 2013). In many cases the faecal coliform levels exceed the WHO guidelines for irrigation of produce (WHO, 1989).

The South African Water Research Commission (WRC) initiated a research project in 2007 with the overall objective of investigating the links between irrigation water quality and food safety in commercial and subsistence agriculture (Dr. G.R. Backeberg, Water Research Commission, Personal communication, 2007). In the Western Cape, fresh produce from the Plankenburg, Mosselbank and Berg Rivers' region is irrigated with river water with high microbial counts (Ackermann, 2010; Lötter, 2010). In previous studies, contributing to the WRC research project, on the Plankenburg and Mosselbank Rivers the faecal coliform counts were found to vary from 1.6×10^5 organisms.100 mL⁻¹ to 4.6×10^5 organisms.100 mL⁻¹, respectively (Lötter, 2010). These results exceeded the DWAF and WHO guidelines of >1 000 *E. coli* per 100 mL water for irrigation of fresh produce (WHO, 1989). In many cases (Bezuidenhout *et al.*, 2002; Obi *et al.*, 2002; Lin *et al.*, 2003; Barnes & Taylor, 2004; Paulse *et al.*, 2007) the quality of the river water is impacted by informal settlements along the river banks. Due to insufficient sanitary facilities, faecal and household waste is often dumped in the river (Barnes & Taylor, 2004) which adds to the pollution of rivers. Additionally, according to Barnes & Taylor (2004) non-operational or badly operated sewage works, informal housing and industrial waste in some cases adds to the pollution load. The presence of *E. coli*, *Salmonella*, *Staphylococcus* and *Listeria* has been reported in the

Mosselbank, Berg and Plankenburg Rivers at unacceptable high levels (Bezuidenhout *et al.*, 2002; Obi *et al.*, 2002; Lin *et al.*, 2003; Paulse *et al.*, 2007; Ackermann, 2010; Lötter, 2010). The presence of faecal coliforms have also been recorded in several other South African rivers including the Mhlathuze in KwaZulu-Natal (Bezuidenhout *et al.*, 2002; Lin *et al.*, 2003), Vuwanie, Mutshindudi, Tshinane, Mutale, Mudaswali and Levubu Rivers in the Northern Province (Obi *et al.*, 2002). Subsequently, the different micro-organisms present in the river waters and the carry over to the crops being irrigated has been established. Fresh produce is thus at risk of hosting pathogenic bacteria that can be transmitted to the consumer (Ackermann, 2010; Lötter, 2010).

The most frequent pathogenic micro-organisms associated with foodborne outbreaks are *Salmonella*, *E. coli*, *Shigella*, *Campylobacter jejuni*, *Clostridium perfringens* and *Listeria monocytogenes* (Batz *et al.*, 2011). *Escherichia coli* has been reported in outbreaks mostly associated with food products of bovine origin but occurrence of outbreaks from fruit and vegetables and other non-bovine foods are however, increasing (Harris *et al.*, 2003). *Escherichia coli* (ETEC and EHEC), *Salmonella* and *Campylobacter* spp., among other bacteria, protozoa and enteric viruses, have been identified on fresh produce (Scharff, 2010). The presence of *E. coli* on fresh produce is considered to be an indication of the presence of faecal matter, given that the intestinal tract of humans and warm blooded animals is considered a habitat for *E. coli*. In general, coliforms are not harmful, but the group does include pathogenic bacteria, of which *E. coli* O157:H7 is only one of many examples (Arnone & Walling, 2007). These pathogens can cause foodborne illnesses, especially if contaminated water is used for the irrigation of fresh produce.

Escherichia coli is an emerging foodborne pathogen (Tauxe, 2002) and the species can be divided into three main groups based on pathogenicity consisting of non-pathogenic commensal *E. coli*, intestinal pathogenic *E. coli* and extraintestinal pathogenic *E. coli*. Intestinal pathogenic *E. coli* (INTEC) cause illnesses in the host's intestinal tract and consist of Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC) and Diffusely Adherent *E. coli* (DAEC). Each of the six types has a different mechanism for interacting with their host and an infective dose which can cause illness in the host. Extraintestinal pathogenic *E. coli* (ExPEC) cause infections outside of the intestinal tract. Extraintestinal pathogenic *E. coli* has recently been classified as a group consisting of three pathotypes; Uropathogenic (UPEC), Sepsis associated (SEPEC) and Neonatal meningitis associated (NEMEC) (Russo & Johnson, 2009). Extraintestinal pathogenic *E. coli* can be ingested together with intestinal pathogenic *E. coli* orally but does not cause disease in the intestinal tract. A variety of virulent factors enable ExPEC, however, to cause infection in other sterile body sites. Thus ExPEC and INTEC are equally threatening to the consumer.

Worldwide, faecal coliforms (*E. coli*) are considered to be an indicator organism of water safety especially in agriculture (Anon., 2003). In 1981, a study by Garcia-Villanova Ruiz *et al.* (1987) on fresh vegetables from farms, a wholesale market, supermarkets and a small shop in Granada, Spain showed a high level of faecal contamination. Of the samples 86% were positive for

the presence of *E. coli* (Garcia-Villanova Ruiz *et al.*, 1987). In another study done using contaminated water for irrigation of spinach and lettuce, pathogens were found to be present. After only two weeks of irrigation *E. coli* O157 was predominant when compared to *Salmonella* and *Campylobacter* (Monaghan & Hutchison, 2008). The study also showed that the pathogens on the produce decreased a week after irrigation with very low counts, too few to count. It was concluded that the time between irrigation and harvest is of importance to the farmer, in order to prevent pathogen presence on produce. In the Eastern Cape, South Africa, a study by Abong'o *et al.* (2008) tested specifically for the presence of *E. coli* O157:H7. The vegetables sampled in the study included cabbage, cucumbers, spinach, onions and carrots from farmers' markets and retail stores in the Amathole District, Eastern Cape. The level of *E. coli* O157:H7 ranged from 1.3×10^3 cfu.g⁻¹ – 1.6×10^6 cfu.g⁻¹ on the vegetables sampled (Abong'o *et al.*, 2008). In the US shredded Romaine lettuce was reported to be the source of an *E. coli* O145 outbreak. This outbreak confirmed at least 26 cases of foodborne infections (CDC, 2010). The most recent outbreak of *E. coli* with fresh produce as the source occurred in Germany in 2011. The culprit strain was *E. coli* O104:H4 linked to fenugreek sprouts (Warriner, 2011). Not less than 4 075 cases of illness were confirmed including 908 cases of haemolytic uraemic syndrome (HUS) and in total 50 people lost their lives (WHO, 2011).

The presence of environmental strains of *E. coli* was reported by McLellan (2004) and Power *et al.* (2005) and these strains were shown to survive and multiply in the environment. Another study was done on soil in a tropical rainforest area and numerous *E. coli* strains were found. There was no sign of faecal contamination near the sampling sites, thus the strains found and identified as *E. coli* were considered not to be of faecal origin (Lasalde *et al.*, 2005). Thus, the conclusion was reached that the presence of *E. coli* might not always be indicative of faecal pollution.

The O104:H4 strain found in Germany (Warriner, 2011) is a good example of a unique *E. coli* strain as it can not be characterised to only one subgroup of intestinal pathogenic *E. coli*. The characteristics of the O104:H4 allow this strain to be characterised as both EHEC and EAEC. The EAEC virulence plasmid was present as well as Shiga toxin 2 (stx2a) which is characteristic of EHEC (Warriner, 2011). *Escherichia coli* is known to be genetically highly adaptable and are able to exchange genes among one another through horizontal gene transfer (Karberg *et al.*, 2011). This could lead to many *E. coli* variations as result. It is thus possible that undiscovered environmental pathogenic *E. coli* strains exist which can easily enter the human food chain through contaminated fresh produce.

The overall objective of this study is to determine the presence, cell numbers and specific types of *E. coli* present on fresh produce. To do this, fresh produce from “point-of-harvest” and “post-harvest” sample sites in the Western Cape will be used. Point-of-harvest sample sites will be from commercial and small scale farms while post-harvest samples will be from retail outlets and farmers' markets. The fresh produce types to be examined will be limited to produce that is

consumed raw by the consumer and will include peas, spinach, beans, cabbage, lettuce, tomatoes, bean sprouts and fresh herbs (mint, basil, parsley, rocket and thyme). The presence of INTEC and ExPEC strains will be determined. A possible risk assessment will be compiled to give an indication of the potential hazard of pathogenic *E. coli* on fresh produce.

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CHAPTER 2 LITERATURE REVIEW

INTRODUCTION

Fresh produce has become a popular part of the human diet due its fresh, tasty and healthy characteristics (Hanif *et al.*, 2006). Consumers in general perceive fresh food to be more healthy and nutritious and are willing to pay more for the fresher food, or anything that is said to be fresh (Anon., 2011a) and as a result salad bars and restaurants are increasing. "Fruit and Vegetables" were on the "Top Ten Nutrition Trends" for 2012 (Anon., 2012) and this can be ascribed to the health aspects of fruit and vegetables and thus forms an important segment of the food industry. Consumers also prefer to buy fresh produce that is locally produced as they feel that locally produced crops are beneficial to the economy, environment and to the quality of the produce. Organic produce is perceived in the same light as local produce (Govindasamy *et al.*, 2002; Tobin *et al.*, 2012).

Fresh fruit and vegetables have been linked to major sources of foodborne outbreaks and these are increasing all over the world (Ackers *et al.*, 1998; Harris *et al.*, 2003; Lynch *et al.*, 2009). This makes it more important to ensure microbial safety of fresh produce especially when used for raw consumption (Garret *et al.*, 2003). Increased outbreaks can ultimately damage the consumer's confidence and beside the infection aspect can also result in consumers changing their eating habits. The latter will lead to a negative economical turn for the fresh produce industry and thus supports the importance of the high investment made in microbiological surveillance (Heaton & Jones, 2008).

Foodborne linked pathogenic outbreaks from vegetables are most probably due to faecal contamination of river water used for irrigation (Okafo *et al.*, 2003). Thus, if present, pathogenic bacteria will attach to the surface of the vegetables but their presence on fresh produce will differ according to the type of produce and prevalent environmental conditions. Some vegetables are consumed raw, which means that if pathogens are present they will not be removed during a washing or even a heat process.

According to DWAF, water safety is evaluated in terms of: the total coliforms which are seen as indicators of general hygiene; the faecal coliforms which are indicators of faecal pollution; and *Escherichia coli* which is considered the specific indicator of faecal pollution (DWAF, 1996b). The latter pollution can lead to foodborne illnesses, especially if the water is used for irrigation of fresh produce. Additionally *E. coli* is used as an indicator of water safety especially in agriculture (Anon., 2003).

In the past the measure of the quality of irrigation water was based on factors like pH, hardness, carbon levels and mineral content, especially since these have an impact on plant health (Anon., 2006). However, it is important to also include microbial safety when determining the

quality of irrigation water. If the microbiological quality of irrigation water is not up to standard, there will be a safety risk to the consumer especially if the fresh produce will be consumed raw.

FOODBORNE DISEASE OUTBREAKS LINKED TO FRESH PRODUCE

Foodborne disease outbreaks are not a recent occurrence as the presence of Gram-negative bacteria on vegetable tissue was already reported more than 40 years ago (Samish *et al.*, 1962). In 1981, a study was initiated to sample fresh vegetables from farms, a wholesale market, supermarkets and small shops in Granada, Spain. The results obtained emphasised the high degree of faecal contamination on vegetables with 86% of the samples testing positive for *E. coli* (Garcia-Villanova Ruiz *et al.*, 1987). Since then, outbreaks as a result of the consumption of fresh produce have increased worldwide (Lynch *et al.*, 2009).

Food and waterborne outbreaks in SA

Information on outbreaks or presence of pathogens in or on fresh produce leading to foodborne outbreaks in South Africa is scarce, partly due to the absence of an efficient reporting system. Over the last few years South Africa has put in place a reporting system operated by the National Institute for Communicable Diseases (NICD), a division of the National Health Laboratory Service (NHLS), to provide up-to-date information on communicable diseases in South Africa. Numerous foodborne illness outbreaks have been reported in the Communicable Diseases Communiqué (NICD, 2011a; NICD, 2011b; NICD, 2012). But still, an evaluation of the fresh produce industry in South Africa in terms of fresh produce contamination needs to be done to determine the scope of the problem and to ensure food safety.

For example, in the Eastern Cape a study by Abong'o and co-workers (2008) tested specifically for the presence of *E. coli* O157:H7 present on cabbage, cucumbers, spinach, onions and carrots from farmers' markets and retail stores. The prevalence of *E. coli* O157:H7 on the vegetable samples was found to range from 1.3×10^3 to 1.6×10^6 cfu.g⁻¹. Recently it was reported that *E. coli* had been detected on fresh produce from retail outlets (Hyslop, 2011). Thus, from the available literature in South Africa it was concluded that there is a concern in terms of foodborne pathogens present on fresh produce.

Examples of outbreaks world-wide

In terms of food and waterborne disease incidences worldwide, diarrhoeal disease has been found to be the most prevalent with 4 620 million incidences occurring per year (WHO, 2004). Foodborne infection leads mostly to diarrhoeal diseases which can severely damage human intestines. The majority of these incidences occurred in South-East Asia and the Western Pacific with 1 276 million and 1 255 million, cases being reported, respectively. This was followed in numbers by Africa, the Americas, Eastern Mediterranean and Europe with 912 million, 543 million,

424 million and 207 million cases, respectively (WHO, 2004). In many cases diarrhoea is downplayed as a mere “stomach bug” but it can in actual fact be a case of a foodborne infection.

The most recent outbreaks reported by the Centres for Disease Control (CDC) on pathogenic *E. coli* include strains O26, O157:H7, O104:H4 and O145. In the USA, eight people were infected during an outbreak in December 2010 with *E. coli* O157:H7. Laboratory testing connected DNA isolates from a patient infected with *E. coli* O157:H7 to in-shell hazelnuts from the same patient's home. From the eight people infected, four were admitted to hospital, but haemolytic uraemic syndrome (Kämpfer *et al.*, 2008) was not detected and there were no deaths (CDC, 2011a). Shredded Romaine lettuce was reported to be the source of an *E. coli* O145 outbreak. The Shiga toxin-producing *E. coli* (STEC) O145 is not frequently reported, thus outbreaks concerning this strain are possibly more prevalent than documented. This was also the first outbreak of this strain in the USA and 26 cases and seven suspected cases were confirmed (CDC, 2010). During December 2011 through to February 2012, 29 people have been infected, from which seven were hospitalised, with Shiga toxin-producing *E. coli* O26 through raw clover sprouts from a restaurant in the USA (CDC, 2012a). An outbreak of Shiga toxin-producing *E. coli* O145 occurred during April 2012 until June 2012 and infected 18 people in the USA with four hospitalised and one dead (CDC, 2012b). Organic spinach and a spring mix blend was linked to an outbreak of Shiga toxin-producing *E. coli* O157:H7 in the USA that caused 13 hospitalisations, two cases of haemolytic uraemic syndrome (HUS) with a total of 33 people infected during October 2011 and November 2012 (CDC, 2012c). This product had to be recalled (CDC, 2012c).

Almost the biggest outbreak of pathogenic *E. coli* occurred recently in Germany and was caused by *E. coli* O104:H4. Initially it was stated that cucumbers from Spain were the source (Anon., 2011b). Later, in June 2011 the source of the outbreak was speculated to be raw sprouts from a farm in Germany (Anon., 2011c). Sprouts are a high risk food and *E. coli* O104:H4 is an extremely virulent pathogen, thus when the two collide it is destined for disaster (Warriner, 2011). The problem with sprouts is that they are germinated at 37°C which is the optimum for *E. coli* growth (Anon., 2011c). Thus, it could be an ideal environment for *E. coli* growth. The German strain was also shown to be a Shiga toxin-producing (STEC) strain. In the latest update from the CDC, in July 2011, the Robert Koch Institute recorded 823 cases of haemolytic uraemic syndrome (HUS) infection from which six have been confirmed to be from STEC O104:H4 origin and ultimately more than 4 000 cases were confirmed (CDC, 2011b; Warriner, 2011). From these 4 000 cases, 44 resulted in death.

This specific Shiga toxin (*stx2* gene) producing *E. coli* O104:H4 strain has been shown to have characteristics identical to Enterohemorrhagic *E. coli* (Warriner, 2011). This strain can also be grouped both as a STEC and an Enteroaggregative *E. coli* (EAEC) strain as it is 93% similar to EAEC, but the presence of *stx2* links it to EHEC (Warriner, 2011). It was interesting to note the presence of the *stx2* gene but the absence of intimin and enterohemolysin as these virulence factors would typically be present together in the same bacteria. The *E. coli* O104:H4 strains have

been shown to be resistant to a range of antibiotics (ampicillin, streptomycin, tetracyclin, cefotaxime, cefazidime, nalidixic acid, trimethoprim/sulfamethoxazol, amoxicillin/clavulanic acid, cefuroxime-axetil, piperacillin/sulbactam, piperacillin/tazobactam, ceftiofur, cefuroxime and cefpodoxime). This is of importance as this makes it difficult to treat a foodborne infection caused by an antibiotic resistant bacterium (Warriner, 2011).

A cluster of *E. coli* O104:H4 infections were also reported in June 2011 in France (CDC, 2011b). This occurrence was at a conference event in Bordeaux where the attendees consumed locally produced sprouts. In July 2011 the European Food Safety Authority reported that the most probable source of the *E. coli* O104:H4 outbreaks in Germany and France were bean sprouts (CDC, 2011b).

In Nigeria a study was done on water from the Kubanni River that was utilised for domestic activities and vegetable (lettuce, tomatoes, cabbage, and spinach) irrigation (Chigor *et al.*, 2010). The pollution of this river water was as a result of pollution from a sewage treatment plant, an abattoir and domestic sewage. Standard methods were used to test for *E. coli*, specifically *E. coli* O157. Two of the 96 water samples examined were positive for *E. coli* O157. Also, it showed that the faecal coliform count was higher in the dry season than in the rainy season (Chigor *et al.*, 2010). Two sites closer to the sewage treatment plant and the abattoir had higher faecal coliform counts, but all of the samples taken still had higher faecal coliform counts than the guidelines of the World Health Organization for irrigation water (WHO, 1989; Chigor *et al.*, 2010). If the faecal coliform counts in river water used for irrigation of fresh produce is higher in the dry season this would indicate a higher risk of contamination from produce produced primarily in the warmer months (Chigor *et al.*, 2010).

Another study done in Nigeria on river water irrigated vegetables, which were tested for contamination (Okafo *et al.*, 2003), distribution over wet and dry seasons and the extent of pathogens isolated. Ultimately 196 water and 326 vegetable samples were tested for coliforms and the presence of *Salmonella*, *Vibrio* and *E. coli* over two seasons (wet and dry seasons). The counts on the vegetables were at levels of more than 10^5 cells of *E. coli* per 1 mL. Again it was found that the numbers both in the water and on the vegetables were more numerous in the dry season than in the wet season. The *E. coli* detected was identified as ETEC and could thus cause diarrhoea if transferred to humans (Okafo *et al.*, 2003).

In another study in the UK, spinach and lettuce irrigated with contaminated water were tested and results showed the pathotypes of *E. coli* O157, *Salmonella* and *Campylobacter* present at levels that were too numerous to count after only two weeks of irrigation. *Escherichia coli* O157 was prevalent compared to *Salmonella* and *Campylobacter* (Monaghan & Hutchison, 2008). The study also reported that the pathogens on the produce were found to decrease a week after irrigation. The time between irrigation and harvest is thus an important factor that must be taken into consideration when studying the risks involved during cultivation of fresh produce (Monaghan & Hutchison, 2008).

Occurrence of pathogenic bacteria on fresh produce

Outbreaks associated with fruits and vegetables have increased over the past decade. *Escherichia coli* (ETEC and EHEC), *Salmonella* and *Campylobacter* spp. and other bacterial pathogens, protozoa and enteric viruses, have also been identified on fresh produce (Scharff, 2010). It is possible that the cases of fresh produce being the source of foodborne pathogens increased as a result of better recognition and reporting systems (Beuchat & Ryu, 1997; Burnett & Beuchat, 2001; Beuchat, 2002). Burnett & Beuchat (2001) also reported that the increase in presence of pathogenic bacteria on fresh produce could be due to processing, harvesting and distribution modifications.

Numerous micro-organisms have been identified on fresh produce that can lead to foodborne-illness (Abadias *et al.*, 2008). Some of these correlate with waterborne pathogens, which is an indication that the use of contaminated irrigation water could result in the transfer of pathogens to produce (Mena, 2006). The microbes that have been identified as water contaminants and that have been shown to be present on irrigated produce are listed in Table 1.

Table 1 Waterborne pathogens found on fresh produce (Brackett, 1999; Rosen, 2000; Okafo *et al.*, 2003; Mena, 2006; Cabral, 2010; Gelting *et al.*, 2011; Ijabadeniyi *et al.*, 2011; Jacobsen & Bech, 2012)

Bacteria	Enteric viruses	Protozoa
<ul style="list-style-type: none"> • <i>Campylobacter</i> spp • <i>Escherichia coli</i> (ETEC and EHEC) • <i>Salmonella</i> • <i>Shigella</i> spp • <i>Vibrio cholerae</i> • <i>Yersinia enterocolitica</i> 	<ul style="list-style-type: none"> • Hepatitis A Virus • Norovirus 	<ul style="list-style-type: none"> • <i>Cyclospora</i> • <i>Cryptosporidium</i> • <i>Giardia</i>

In another study in Norway markets were tested for their bacterial quality and potential risk to the consumer. The data showed a risk of foodborne diseases to the consumer as *Yersinia enterocolitica* and *Listeria monocytogenes* were detected on nine of 890 fresh produce samples (Johannessen *et al.*, 2002). Another study was done by Tian *et al.* (2012) to determine the survival and/or growth of pathogens on vegetables at average storage temperatures of 4° to 15°C. Different combinations were used with four different types of strains and three types of lettuce leaf and sprouts at different time intervals. The growth results differed for all the combinations tested, although all of the strains survived. *Escherichia coli* O157:H7 and *S. typhimurium* on lettuce increased with a maximum of 2 log cfu.g⁻¹ after 1 day at 15°C (Tian *et al.*, 2012). In another study, vegetables from retail outlets in Spain were tested to determine microbial contamination levels and

if any pathogenic bacteria were present (Abadias *et al.*, 2008). The results did not show any foodborne pathogens, but the possibility of them occurring was indicated, as 40% of the sprouts tested positive for *E. coli* and 14.8% of the other vegetables were positive for *E. coli*, 1.3% positive for *Salmonella* and 0.7% positive for *L. monocytogenes* (Abadias *et al.*, 2008).

Organic vegetables were tested for the presence of species of *Aeromonas* spp., *Salmonella*, *Campylobacter*, *Escherichia* and *Listeria*. Only *Aeromonas* spp. was found on 41% of the 86 samples tested (McMahon & Wilson, 2001). A similar study was done in Nigeria where it is common for small-scale crops, grown for local urban markets, to be irrigated with water from rivers that had been used for waste disposal (Dreschel *et al.*, 2006). The presence of species of *Salmonella*, *Vibrio* and *E. coli* were found of which 39 isolates were Enteropathogenic *E. coli* (Okafo *et al.*, 2003).

Vegetables irrigated with water from the Litani River in Bekaa Valley, Lebanon, were tested for microbiological quality. The vegetable samples included lettuce, parsley and *Malva*. The lettuce had the highest counts, where *E. coli* was present on 42.3% of the lettuce and on 13.8% of the parsley samples. *Staphylococcus aureus* was detected on 51.5% of the lettuce and on 38% of the parsley samples (Halablab *et al.*, 2011).

Zhang *et al.* (2009) found that *E. coli* O157:H7 can survive for a longer period on the inside of a lettuce leaf, compared to the outer leaf surface. This can result in internalisation which means that simply washing the lettuce after harvest will not remove all the pathogens (Aruscavage *et al.*, 2006). Therefore, if fresh produce is contaminated with pathogenic bacteria they will not be efficiently removed by the consumer.

SOURCES OF CONTAMINATION

Background

Fresh produce can become contaminated at any production stage from the farm to the retail store. Contamination sources can include faeces, soil, insects, dust, irrigation water, inadequately composted manure, animals, human handling, processing equipment, transport vehicles, transport containers and harvesting equipment (Beuchat & Ryu, 1997; Beuchat, 2002). Johnston *et al.* (2006) reported that *E. coli* loads can increase by $2 \log \text{ cfu.g}^{-1}$ on cantaloupe after harvest and during processing. It can also get contaminated from the retail outlet to the consumers home, but if this is the case it is the consumers own responsibility. Advice to the consumer to ensure clean produce will be to wash the fresh produce thoroughly. In some cases this will however not be sufficient as certain pathogenic bacteria have the ability to attach to the surface of the produce in such a manner that washing will not result in removal (Critzler & Doyle, 2010).

Escherichia coli are found in the intestinal gut of humans and other warm-blooded animals. Thus, almost all contamination of *E. coli* can be traced back to faecal matter as the original source. This can also result in almost anything becoming contaminated with *E. coli*. Fresh produce

specifically can be contaminated through soil, irrigation water and handling during harvest, processing and packaging (Brackett, 1999; Beuchat, 2002; Farrar & Guzewich, 2009). A study done in Germany concluded that the prevalence of *E. coli* on fresh produce is relatively low due to synthetic fertilisers being used and not manure (Schwaiger *et al.*, 2010). The latter statement can be disregarded if the irrigation water used for the fresh produce is contaminated with manure or sewage waste (Schwaiger *et al.*, 2010). Harvesting equipment should be cleaned regularly and the workers handling the fresh produce, in the field and packinghouse, should also be well-informed about maintaining good hygiene (Beuchat & Ryu, 1997; Brackett, 1999). Manure should not be used in soil where produce is grown that will be consumed raw. Water used for irrigation should be tested regularly for faecal contamination (Monaghan & Hutchison, 2008). A possible source of contamination that is not typically recognised is the handling included during the distribution phase i.e. cold chain maintenance, loading by dock workers and truck drivers (Brackett, 1999; Lynch *et al.*, 2009).

River and irrigation water

In South Africa water scarcity is an economic reality (Turton, 2008) and has a direct impact on the agricultural sector especially when polluted water is used for irrigation. Since a river can flow through various locations different forms of pollution may occur additionally. River pollution can be categorised as either a point-source or a non-point-source (Stewart *et al.*, 2008). Point-source is a specific point that can be identified, measured and controlled. Non-point-source is not measurable and unidentifiable (Stewart *et al.*, 2008). People living near rivers also sometimes use it to dump household waste into, which can include biological and chemical waste (Barnes & Taylor, 2004).

In South Africa water from a nearby river is often used for irrigation of fresh produce. This irrigation water, however, is often polluted with high microbial levels (Ackermann, 2010; Lötter, 2010; Van Blommestein, 2012; Britz *et al.*, 2013). As a result the South African Water Research Commission started a research project in 2007 to monitor the microbial types and loads present in river waters and the degree of carry-over to the crops being irrigated (Dr. G.R. Backeberg, Water Research Commission, Personal communication, 2007). The microbial types reported includes coliforms, faecal coliforms and *E. coli* as “Indicator organisms”, and *Staphylococcus*, *Salmonella*, *Listeria* and intestinal *Enterococci* as “Index organisms” (Ackermann, 2010; Lötter, 2010).

Available literature on microbial contamination in South African rivers includes studies done on fresh produce and river water used for crop irrigation. The presence of *E. coli*, *Salmonella* and *Listeria* has been reported in the Mosselbank, Eerste, Berg and Plankenburg Rivers in the Western Cape (Paulse *et al.*, 2007a; Paulse *et al.*, 2007b; Ackermann, 2010; Lötter, 2010; Van Blommestein, 2012). The faecal coliform counts were found to vary from 160 000 to 460 000 organisms.100 mL⁻¹, respectively (Lötter, 2010). These results exceed the DWAF guidelines of less than 1 000 *E. coli* per 100 mL water for irrigation of fresh produce (DWAF, 1996a). Faecal coliforms have also been detected in the South African rivers like the Mhlathuze River in KwaZulu-

Natal (Bezuidenhout *et al.*, 2002; Lin *et al.*, 2004), and the Vuwanie, Mutshindudi, Tshinane, Mutale, Mudaswali and Levubu Rivers in the Northern Province (Obi *et al.*, 2002). The presence of coliforms and *E. coli* was also reported in Baynespruit River in Sobantu, a sub-urban community in KwaZulu-Natal (Gemmell & Schmidt, 2012). The loads detected again exceeded the WHO guidelines (WHO, 1989). This river is used to irrigate fresh produce which was tested as well to determine the carry-over effect. The results from this study indicated carry-over of coliforms and *E. coli* with loads that exceeded the Department of Health (DoH) guidelines for safe consumption (DoH, 2011).

Other studies done in South Africa showed the following pollution levels: Berg River in the Western Cape ranged from 1 600 – 35 000 000 faecal coliforms.100 mL⁻¹; Renoster Spruit in Bloemfontein ranged from 4 870 – 59 000 *E. coli*.100 mL⁻¹; Zandvlei, Zeekoeivlei and Princess Vlei in the Cape Flats ranged from 1 000 – 100 000 faecal coliforms.100 mL⁻¹ and the Mhlathuze River in KwaZulu-Natal ranged from 0 – 27 000 cfu.mL⁻¹ (Harding, 1993; Bezuidenhout *et al.*, 2002; Griesel & Jagals, 2002; Lin *et al.*, 2004).

Soil

Soil can also be a source of contamination that can play a role in depositing thermotolerant coliforms and *E. coli* onto fresh produce plants. Fields previously used for animal grazing will most probably show presence of pathogenic bacteria in the soil. The latter can also be due to flood waters from an area where animals have been grazing (Brackett, 1999). A range of human pathogens including *Clostridium perfringens*, *C. botulinum*, *L. monocytogenes*, *Bacillus cereus* and *Aeromonas* are commonly found in soil (Beuchat & Ryu, 1997; Whipps *et al.*, 2008) and can be expected to be present on fresh produce once in a while. When soil is moist it provides bacteria and viruses with an ideal environment for survival and growth. The soil also has the necessary nutrients, temperature, pH and organic matter that the microbes need to reproduce and survive (Cools *et al.*, 2001; Santamaría & Toranzos, 2003).

The fact that *E. coli* are present in soil adds the possibility of internalisation of *E. coli* into plants (Solomon *et al.*, 2002). The presence of unique environmental strains has been established by studies done in 2004 and 2005 by McLellan (2004) and Power *et al.* (2005) respectively. The data showed that these unique strains were able to survive and multiply in the environment (McLellan, 2004; Power *et al.*, 2005). Thus, the presence of these may be due to the environmental conditions of soil resembling the mammalian intestinal gut in terms water, temperature and warm air (Johnson & Russo, 2002).

In a study on the survival of bacteria in soil after irrigation it was found that *E. coli* O157 was more numerous than *Salmonella* and *Campylobacter* (Monaghan & Hutchison, 2008). According to Beuchat (2002), contamination can occur at any stage from growing through to harvest, post-harvest handling and distribution. A pathogen can adapt to a stressful environment and might become more virulent. Islam *et al.* (2005) reported that *E. coli* O157:H7 can survive in

soil for up to 196 days and on carrots and onions for up to 168 days. This highlights the importance of ensuring the product is safe after every production stage. It is important to establish the source of possible and existing contamination to take measures in eliminating the cause of the contamination.

In a study on soil in a tropical rainforest area numerous *E. coli* strains were found (Lasalde *et al.*, 2005). The researchers found no sign of faecal contamination near the sampling sites, thus the strains found and identified as *E. coli* were not from faecal origin. The presence of such strains is an indication that unique environmental strains can exist without faecal pollution having taken place and thus the environment provides sufficient nutrients, air, temperature and soil for *E. coli* to propagate (Winfield & Groisman, 2003).

ESCHERICHIA COLI AS INDICATOR ORGANISM

An indicator organism can be defined as a single or a group of micro-organisms that will be indicative of the strong possibility of the presence of pathogenic micro-organisms. Testing for an indicator organism is commonly used to assess hygienic conditions (Busta *et al.*, 2003) and microbiological safety of water (Balzer *et al.*, 2010). An indicator organism can be a virus, bacteria or even protozoa (Wen *et al.*, 2009). Pathogenic microbes can cause numerous diseases including cholera, gastroenteritis, typhoid fever, salmonellosis, hepatitis and dysentery. These diseases are commonly the result of contact with contaminated water, drinking water or raw food that was in contact with contaminated water (DWAF, 1996b). It is however possible that an indicator organism can be present without a potential pathogen being present but it is not considered a good indicator organism when this is the case.

The World Health Organization (WHO, 2001) recognises three groups that can be used to indicate microbial water quality: general microbial indicators; faecal indicators; and index and model organisms. General microbial indicators give an indication of the effectiveness of a process, while faecal indicators indicate the presence of faecal pollution and index and model organisms give an indication of the presence of pathogens (Ashbolt *et al.*, 2001).

For an organism to be a good indicator it should have similar properties and behaviour to the species it is indicative of. For indicator organisms to be representative of pathogens they should fulfil the following criteria (DWAF 1996a):

- Be applicable for all water types;
- Be present in contaminated waters together with pathogens;
- Be present in loads that link with the extent of pollution;
- Occur in higher loads than those of the pathogens;
- Not reproduce in the water environment;
- Be able to survive as long as pathogens in the environment;
- Not be present in non-contaminated water;
- Be detectable with practical and reliable methods; and

- Be safe to work with in the laboratory and be non-pathogenic.

The reason for *E. coli* being the best indicator organism for indication of organisms of faecal contamination is that it is dominant in the gastrointestinal tract in all warm-blooded animals including humans. Thus, if *E. coli* are present, it is an indication that water or the sample tested are contaminated with faecal matter and will most probably contain pathogens detrimental to human health. The same is true for vegetables; if faecal coliforms and *E. coli* are present it is safe to assume faecal contamination. Although this is true, contamination can also be due to other sources.

Coliforms consist of *Klebsiella*, *Serratia*, *Hafnia*, *Citrobacter*, *Escherichia* and *Enterobacter* which all grow at 35° - 37°C (Brenner *et al.*, 2005). Faecal coliforms can grow at temperature up to 44°C (Teplitski *et al.*, 2009). The best indicators of faecal contamination for water were coliforms, faecal coliforms and then ultimately *E. coli* (Edberg *et al.*, 2000; Tallon *et al.*, 2005). A study done in Norway to test for the microbiological safety of fresh produce it was concluded that *E. coli* is a better indicator organism than thermotolerant coliforms (Johannessen *et al.*, 2002). This is relevant to South Africa as fresh produce is known to be irrigated with polluted water and thus the water quality is an important safety factor.

ESCHERICHIA COLI CHARACTERISATION

Escherichia coli were first discovered by Theodor Escherich in 1885 who named it *Bacterium coli*. In 1919 the genus was changed to *Escherichia* with main species *Escherichia coli* (Escherich, 1988) as a member of the *Enterobacteriaceae* family. The phenotypic characteristics include rod shaped, non-spore forming, Gram-negative, motile and a facultative anaerobe that produces gas and acid from fermentable carbohydrates (Percival *et al.*, 2004). *Escherichia coli* strains can be grouped according to different characteristics including: phenotypic - the physical expression of a gene; phylogenetic - based on their environmental niches; and pathogenic – a tendency to cause infection (Gordon *et al.*, 2008; Carlos *et al.*, 2010) and serogrouping according to the antigens present on the surface of the bacteria (Bhunia, 2008).

Most *E. coli* strains are not pathogenic and are categorised as commensal *E. coli*. In contrast *E. coli* O157:H7 and *E. coli* O104:H4 are two examples of pathogenic strains (Arnone & Walling, 2007). The possibility of non-pathogenic environmental strains has also been reported (McLellan, 2004; Power *et al.*, 2005). A study done on soil from a tropical rain forest, with faecal contamination as source eliminated, found the presence of numerous *E. coli* strains (Lasalde *et al.*, 2005).

Escherichia coli have the ability to exchange genes and this characteristic will result in various pathogenic strains. The genes are not only exchanged between *E. coli* strains but also between other *Enterobacteriaceae* members (Karberg *et al.*, 2011). For example the O104:H4 strain found in Germany is a good example of a unique *E. coli* strain as it can not be placed in only one group of intestinal pathogenic *E. coli*. Since the EAEC virulence plasmid is present as

well as Shiga toxin 2 which is characteristic of STEC (Struelens *et al.*, 2011; Uyttendaele *et al.*, 2011; Warriner, 2011). These characteristics allow this strain to be characterised as both an STEC and an EAEC.

Commensal *E. coli*

The term commensal *E. coli* is used to describe the group of non-pathogenic *E. coli* strain (Cooke, 1974; Bhunia, 2008). In a way this can be referred to as original or basic *E. coli*. Commensal *E. coli* differ from pathogenic *E. coli* strains in the sense that virulent genes are not present, thus they can not cause infections. It has been shown that their non-pathogenic framework is the same; the difference is in their genes (Ingerson-Mahar & Reid, 2011). Commensal *E. coli* are not harmful to humans and are prevalent in humans' and other warm-blooded animals' intestinal tract where they are beneficial to the health of their host (Cooke, 1974; Bhunia, 2008). However, if they end up in the wrong location in the body, even these commercial *E. coli* can cause infection.

Phylogenetic groupings

Phylogenetic or genotypic grouping is based on the presence in a specific environmental niche and a tendency to cause infection (Gordon *et al.*, 2008; Carlos *et al.*, 2010). *Escherichia coli* can be divided into four phylogenetic groups (A, B1, B2 and D) (Lecointre *et al.*, 1998; Gordon & Cowling, 2003; Gordon *et al.*, 2008; Carlos *et al.*, 2010) and can further be divided into seven sub-groups: A₀, A₁, B1, B2₂, B2₃, D₁ and D₂ (Carlos *et al.*, 2010). Strains in each of the four groups will differ according to their phylogenetic characteristics. These consist of their genome size, their antibiotic resistance profiles and how they utilise different carbohydrates. These characteristics are ultimately a result of the DNA and thus they have different genes that encode these characteristics (Lecointre *et al.*, 1998; Gordon *et al.*, 2008).

The three types of *E. coli* (commensal *E. coli*, intestinal *E. coli* and extraintestinal *E. coli*) can be grouped roughly into the phylogenetic groups in terms of the environmental niche and plasmids present. The commensal *E. coli* are most often placed in the A and B1 groups and it is known not to have plasmids present that contain virulence genes (Johnson *et al.*, 2001). Intestinal pathogenic *E. coli* strains are placed in the phylogenetic groups A, B1 and D (Pupo *et al.*, 1997) while the ExPEC cluster in phylogenetic group B2 and on occasion in group D (Johnson *et al.*, 2001).

Serotypes

Every *E. coli* strain has its own sero 'name' e.g. *E. coli* O157:H7 and *E. coli* O104:H4. The name of each specific *E. coli* strain is based on the three types of antigens present on the surface of the bacterium. The "O" antigens consist of lipopolysaccharide (LPS). There are 174 O antigens and they are numbered from 1 – 181, with the numbers 31, 47, 67, 72, 93, 94 and 122 not assigned. The "H" antigens are known as flagellar antigens and are numbered from 1 – 53 with strains that

do not have flagella being non-motile (Aneck-Hahn *et al.*, 2009). The third antigen is the “K” antigen or capsular antigen. The “O” antigen identifies the serogroup to which the *E. coli* strain belong and the “H” antigen and/or “K” antigen identifies the serotype (Kaper *et al.*, 2004; Bhunia, 2008). For example *E. coli* O157:H7 will be of serogroup O157 and of serotype H7.

Pathotypes

A pathotype can be defined as an organism that could cause a disease (Bhunia, 2008). *Escherichia coli* can be divided into three groups, the commensal *E. coli*, intestinal *E. coli* and extraintestinal *E. coli*. Commensal *E. coli* are non-pathogenic while the intestinal pathogenic *E. coli* may cause illnesses in the intestinal tract of the human. These consist of Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC) and Diffusely Adherent *E. coli* (DAEC) (Donneberg & Kaper, 1992; O'Brien & Holmes, 1996; Scaletsky *et al.*, 2002; Kaper *et al.*, 2004). Each of the six types has a different mechanism for interacting and infecting their host and an infective dose which could have a negative health impact.

Extraintestinal pathogenic *E. coli* (ExPEC) cause infections outside of the intestinal tract. Extraintestinal pathogenic *E. coli* has been classified as a group of pathotypes consisting of three pathotypes; Uropathogenesis (UPEC), Sepsis associated (SEPEC) and Neonatal meningitis associated (NEMEC) (Johnson *et al.*, 2001; Johnson & Russo, 2002; Russo & Johnson, 2009).

ETEC - Enterotoxigenic *E. coli* produce two toxins, heat-labile (LT) which is a large oligomeric enterotoxin and heat-stable (ST) which is a short polypeptide chained toxin (Percival *et al.*, 2004; Bhunia, 2008). These toxins are known to cause infectious diarrhoea in humans. Each of these two toxins has 2 types; LT-I, LT-II, STa and STb. LT-I is similar to the cholera toxin, in terms of genetic grouping, and the symptoms produced are similar to *Vibrio cholerae*. Disease caused by LT-I expressed *E. coli*, largely occurs in animals and humans and disease caused by LT-II expressed *E. coli* occur primarily in animals (Bhunia, 2008).

STa is soluble in methanol and isolated from humans. STb is a methanol insoluble toxin that is isolated from pigs (Bhunia, 2008). STb is expressed only in porcine ETEC strains but it is possible for some human ETEC strains to produce STb. Both these toxins lead to extensive water loss (Bhunia, 2008).

The infective dose of ETEC is $10^6 - 10^9$ organisms (Percival *et al.*, 2004). This pathotype is responsible for gastroenteritis with copious watery diarrhoea with abdominal cramps and vomiting with fever manifested in a small fraction of patients (Percival *et al.*, 2004; Qadri *et al.*, 2005). ETEC is known to cause traveller's diarrhoea in people that are not originally from tropical countries. People from tropical countries become asymptomatic carriers due to being mucosal immune. Traveller's diarrhoea is caused by ETEC found in water which contributes 2 – 8% of the total *E. coli* found in water (Percival *et al.*, 2004; Qadri *et al.*, 2005).

EPEC - Enteropathogenic *E. coli* cause diarrhoea but not due to toxin production. The course of action consists of attaching and effacing lesions on the surface of intestinal cells (epithelial cells) (Garmendia *et al.*, 2005). This leads to wiping out the microvillus on the surface and will result in less nutrients being absorbed (Bhunias, 2008). *E. coli*, with fimbriae, attach to intestinal enterocyte cells. This causes assorted extracellular proteins to be secreted that terminate the microvilli (Bhunias, 2008).

The pathogenesis of EPEC consists of four stages with expression of adhesion factors; followed by initial localised adherence, signal transduction and intimate contact and then cytoskeletal rearrangement and pedestal formation (Bhunias, 2008). The expression of adhesion factors starts when bacteria bind to the epithelial cells. Type IV adhesion fimbriae mediates adhesion and this fimbriae is known as bundle-forming pili (BFP) (Falkow, 1996). BFP is also known as the EPEC adherence factor (EAF).

Signal transduction in the host cell is activated by EPEC attaching to mammalian cells. The signal transduction pathways are responsible for activating the host cell's "tyrosine kinase". Attaching and effacing (A/E) lesions are formed due to abnormalities in the cytoskeletal structure. These abnormalities are caused by broad rearrangement of actin. At 37°C, A/E activity and production of BFP is optimal and grow exponentially. Effacement defines the loss of microvilli. Beneath the bacteria, the host cell membrane forms a pedestal-like structure because of the immense cytoskeletal rearrangements in the host cell (Bhunias, 2008). Pedestal formation is the result of intimate contact. The membrane permeability is increased together with the tight junction (Schets *et al.*, 2005) proteins and mitochondrial function being affected. These reactions result in a malabsorption of nutrients and ions, cell death and ultimately the onset of osmotic diarrhoea.

The infective dose is as high as $10^8 - 10^{10}$ organisms. Thus, high EPEC loads must be present in the human system to cause infection. Infections caused by EPEC are transferred from person to person. This pathotype is responsible for infant watery diarrhoea infections, mostly in developing countries. The mortality rate can go up to 30% (Percival *et al.*, 2004).

EIEC - Enteroinvasive *E. coli* is similar to *Shigella* spp in terms of pathogenicity (Hultgren *et al.*, 1996; O'Brien & Holmes, 1996; Percival *et al.*, 2004). EIEC binds and then invades epithelial cells where it then multiplies intracellularly in the cytoplasm. EIEC then move through the cell directionally and penetrate adjacent cells to allow distribution from cell-to-cell. Invasion of the cells and spreading from cell-to-cell cause extensive cell damage and result in a strong inflammatory response and bloody mucoid diarrhoea which resembles the bacillary dysentery that is caused by *Shigella*. The disease can also be spread by humans (Bhunias, 2008).

The infectious dose for EIEC is between 10^6 and 10^{10} organisms, this is accepted to be rather high, meaning that more than a few organisms are needed to cause an infection in humans.

The symptoms caused by EIEC include watery diarrhoea and sometimes a small percentage develops bloody diarrhoea (Percival *et al.*, 2004).

EHEC - *Escherichia coli* strains that produce Shiga-like toxin are known as STEC. This toxin is cytotoxic to Vero cells, therefore this pathotype is also known as Vero cytotoxigenic *E. coli* (VTEC) and are primarily associated with *E. coli* O157:H7 (Percival *et al.*, 2004; Garmendia *et al.*, 2005; Bhunia, 2008). This strain does not ferment sorbitol and it does not have β -glucuronidase activity and grows rapidly at 30° - 42°C and poorly at 44° - 45°C. EHEC is grouped under STEC as there are pathotypes other than EHEC producing Shiga-like toxin. The Shiga-like toxin is encoded by the *stx* gene. EHEC contains LEE and A/E activity. This pathotype does not express BFP activity, the homologue of the *lifA* gene encoding lymphostatin is plasmid carried and the TTSS is used (Bhunias, 2008).

EHEC colonises in the intestine when it reaches the intestine from contaminated water or food. Due to *E. coli* O157:H7 being acid resistant, it is able to reach the small intestine through the stomach without being harmed. Acid resistance is improved when cells have previously been exposed to acidic foods like apple cider and fermented salami. These foods contain mild acid and this aids the bacteria to be more resistant to a low pH and results in a better survival through the stomach (Bhunias, 2008).

For infection from EHEC to take place, 100 organisms or more are needed and the infection can last 1 to 12 days (Percival *et al.*, 2004). The negative health impact due to an infection from EHEC includes watery diarrhoea with vomiting, haemorrhagic colitis and haemolytic uraemic syndrome (Kämpfer *et al.*, 2008). Haemorrhagic colitis and HUS are unique to this pathotype. HUS can include reactions of renal failure, microangiopathic, thrombocytopenia and haemolytic anaemia. Haemorrhagic colitis results in revoltingly bloody diarrhoea (Percival *et al.*, 2004). EHEC infections include symptoms like a colicky abdominal pain, diarrhoea and vomiting in some cases that is so severe it becomes bloody (Percival *et al.*, 2004).

EAEC - This pathotype adheres to the host cells and produce its own unique ST toxin known as Enteroaggregative ST (EAST) (O'Brien & Holmes, 1996; Bhunia, 2008). EAEC is known to cause diarrhoea in humans; it can be watery, inflammatory and continual (Okeke & Nataro, 2001). The infectious dose is not known but it has been reported to be high (Percival *et al.*, 2004).

Two types of toxins are produced by EAEC. These include ST-like toxin, also known as Enteroaggregative ST (EAST), and haemolysin-like *E. coli* toxin. The latter is an exotoxin that forms pores. This is similar to haemolysin except that it does not have the ability to lyse red blood cells (Bhunias, 2008).

Epithelial cells are not invaded by EAEC; they do however produce a cytotoxin that is responsible for effects on tissue. The toxin also causes inflammation, recognised by penetration of

mononuclear cells to the submucosa. Humans with a weak immune system are more susceptible to EAEC. Mucoïd will be present in faeces as the diarrhoeal cycle proceeds (Bhunia, 2008).

DAEC - DAEC adhere to Hep-2 cells by a diffusely adherence and intervened by a fimbrial adhesion, similar to EAEC. The gene responsible for fimbriae is found in a plasmid or in a chromosome (Bhunia, 2008) but the manner in which this takes place is not clearly understood. The result of this pathogenesis is diarrhoea in humans, mostly in children older than 12 months (Gunzburg *et al.*, 1993; Scaletsky *et al.*, 2002). The infectious dose for this pathotype is not known but is noted that it is the cause of childhood diarrhoea (Percival *et al.*, 2004).

ExPEC - Extraintestinal pathogenic *E. coli* (ExPEC) is the third group of pathogenic *E. coli*. ExPEC is the cause of many diseases occurring outside of the intestinal tract (Johnson & Russo, 2002; Russo & Johnson, 2003). The groups were previously known individually as uropathogenic *E. coli* (UPEC), sepsis associated *E. coli* (SEPEC) and neonatal meningitis associated *E. coli* (NEMEC) and is now termed ExPEC. ExPEC also includes other strains that can cause a number of other infections outside of the intestinal tract (Johnson & Russo, 2002; Russo & Johnson, 2009). Similar to commensal *E. coli* strains, ExPEC are present in the intestinal tract and do not contribute to the cause of gastroenteritis in humans (Russo & Johnson, 2003).

The organism will enter an extraintestinal region like the urinary tract or the lungs and cause an infection (Russo & Johnson, 2003). Areas that can be infected by ExPEC include urinary tract, abdominal and pelvic sites, lungs (pneumonia), surgical sites as well as the central nervous system (meningitis) (Russo & Johnson, 2003). Since ExPEC have not been researched sufficiently, infectious doses for the different groups of pathogenic *E. coli* strains are not known.

MICROBIAL RISK ASSESSMENT

In the food industry it is important to produce a food product that is free from anything that can be a risk to the consumer's health (Bell & Kyriakides, 2002). This must include safety in terms of physical, chemical and biological hazards. A physical hazard can be described as foreign objects in a food product while a chemical hazard will be any food allergens or a chemical that is toxic to human health (WHO, 2006). A microbiological hazard is a micro-organism that is able to enter the food system at any point, where a physical or chemical hazard will enter the food at a certain step in the production process (Griffith, 2012). It is important to note that a microbiological hazard is different from other hazards in the sense that it can multiply and spread compared to physical or chemical hazards that will not spread or multiply (Griffith, 2012).

A risk can be defined as the probability of a hazard causing a negative health effect and the extent thereof (Griffith, 2012). A risk can further be categorised as qualitative and quantitative. A qualitative risk is the expression of the risk e.g. the risk of pathogenic *E. coli* present on vegetables is a high risk. A quantitative risk is a mathematical statement that is more specific regarding the

risk e.g. 5% of vegetables from retail stores are possibly contaminated with pathogenic *E. coli* (Griffith, 2012). In terms of microbial risks the focus is on the biological safety of food products, specifically microbiological safety. The starting point for ensuring a biologically safe food product will begin with a risk analysis.

A risk analysis consists of risk assessment, risk management and risk communication (WHO, 2006; Griffith, 2012). A risk assessment is the first step in risk analysis. It is the scientific method for identifying and measuring the risk. Basically it will indicate if there is a risk, how likely it is of occurring and what the effect will be. Risk management is a continuous structure to control the reduction or elimination of a hazard. This is done by implementing regulations and critical specifications for the specified food product (WHO, 2006). Risk communication is the information concerning the risk being communicated to all the interested parties involved in ensuring food product safety within the production chain (WHO, 2006). The focus here will be specifically on microbiological risk assessment. Microbiological risk assessment will only focus on biological hazards. Ultimately a microbiological risk assessment will be an essential aid to a HACCP plan to ensure safety of food products for the consumer (Roberts, 2000; Griffith, 2012). Microbiological risk assessment is a process based on science. It consists of hazard identification, hazard characterisation, exposure assessment and risk characterisation (Lammerding & Fazil, 2000; Griffith, 2012).

The risk of fresh produce being the source of a pathogen outbreak has not previously been considered as a logical hypothesis (Lynch *et al.*, 2009). Today it is considered as an equal possibility to bovine sources. The risk of disease outbreaks due to foodborne pathogens in South Africa is not reported as extensively when compared to the reporting system in the United States, by the Centres for Disease Control and Prevention (CDC). Thus it is not feasible to make accurate conclusions about foodborne disease outbreaks in South Africa at present.

Risk assessments have been done in various manners in the food industry with regard to the safety of fresh produce. Risk assessment models have been applied to identify the risk of raw consumption of vegetables irrigated with potentially polluted water (Stine *et al.*, 2005; Hamilton *et al.*, 2006a; Hamilton *et al.*, 2006b). It is done by using a formula to estimate the risk involved. Factors like pathogen concentration in water, crop type, water treatment effectiveness, method of irrigation and time between irrigation and harvest, the pathogens die-off rate after harvest and the amount of produce consumed is taken into account in the formula used to estimate the worst-case scenario (Walls & Scott, 1997; Petterson *et al.*, 2001; Suslow *et al.*, 2003; Stine *et al.*, 2005; Hamilton *et al.*, 2006a; Hamilton *et al.*, 2006b). Hamilton *et al.* (2006a) reported that different ethnic groups are at a similar level of risk even though they might have different eating habits and thus consume vegetables in different amounts. A decision support tool, Recycled Water Irrigation Risk Analysis (RIRA), has been developed in the form of a software program. It calculates the risk of water used for irrigation on crops (Hamilton *et al.*, 2007). This tool can be adapted to different situations as every situation will have its own specified parameters.

To prevent pathogen outbreaks associated with fresh produce there are certain areas according to Lynch *et al.* (2009) to be monitored that can present risk. This includes the quality of the water being used throughout the production process. Faecal contamination of any kind should be avoided as far as possible. Faecal contamination can easily occur on fresh produce before harvest from both animal and human faeces. The washing of fresh produce is currently the only method for sanitising and washing. This might not always be sufficient for produce to be consumed raw. People handling the produce by hand should also be taken into consideration. The latter should not be infected with pathogens or be ill (Lynch *et al.*, 2009). Export of fresh produce will increase the risk of contamination as the produce has a longer distribution distance and thus it might go through more channels before arrival at its final destination (Garret *et al.*, 2003; Harris *et al.*, 2003).

A systematic review has been published that indicates the risk factors involved for microbial contamination of vegetables before harvest (Park *et al.*, 2012). Park reported that the most efficient target for the elimination or reduction of microbial contamination of produce includes contaminated soil and irrigation water. Any possible source of contamination, as previously discussed, should be taken into consideration. A similar suggestion was made that evaluation measures should be implemented as part of every food safety microbial risk assessment from pre-harvest to post-production. Thus, not only should reporting systems and detection methods be in place, but also preventative measures as a standard should be regulated (Garret *et al.*, 2003; ICMSF, 2006).

Consumers prefer products that have been approved by a standard according to the food industry and this adds to the importance of evaluating products thoroughly to prevent foodborne outbreaks (Vermeulen & Biénabe, 2010). A study done to investigate the possibility of irrigation water being the source of an *E. coli* O157:H7 outbreak of spinach suggested that the investigation of any foodborne outbreak should be expanded (Gelting *et al.*, 2011). Thus it is safe to conclude that it is of great importance to have systems in place all over the world that will guarantee efficient investigation and prevention of foodborne pathogens in every production chain.

ECONOMIC IMPACT OF FRESH PRODUCE CONTAMINATION

Background

The world economy has a large impact on basically every aspect in the food industry. Fresh produce is a primary food product that contains numerous minerals and vitamins needed for healthy body functioning. If this fresh produce is unavailable, a large percentage of healthy and affordable food will not be available. Thus it is an important part of the economy as the food industry is a prime aspect for human existence. The economic losses that can occur as a result of fresh produce contamination can be significant and should be taken into consideration. When, for instance, an outbreak on lettuce occurs, every single lettuce on the shelf will have to be removed.

This will lead to money going to waste (Roberts, 2000). Firstly the source of the contamination will have to be determined; all of the recalled product will have to be destroyed and it will have to be replaced with new lettuce. The consumer will be hesitant to buy any related product even if the specific product was not initially contaminated (Palma *et al.*, 2010). Worst-case scenario could be an outbreak occurring with more than one food product at the same time. This could have a severe negative effect on the food industry as a whole.

Agriculture

It has been reported that commercial agriculture is responsible for national food security (Vink & Van Rooyen, 2009). It is not a new concept and thus it can be concluded that agriculture has a direct impact on food risks and then ultimately on the economy. In 2007 the gross farming income for commercial farms was R 79 544 million from which field crops make out 20%, estimated at R 15 909 million (STATSSA, 2007). The contribution of agriculture to the SA economy increased from R 38 billion to R 66 Billion in 2009 (GCIS, 2011). The limiting factor of agricultural production that is of most concern is water availability. The fact that water pollution is a reality in SA makes this even more limiting. Run-off water in South Africa is mostly used for irrigation in agriculture; 90% is used for irrigation of fruit, vegetables and wine in South Africa (GCIS, 2011).

Vegetables – economic values

In South Africa the vegetable prices increased from 2008 to 2009 by 42.3%. The income from vegetables (from 2008 to 2009) increased by 19.3%, from R 9 972 million to R 11 901 million (GCIS, 2011). This is a big amount of money that can go to waste if an outbreak occurs. The gross income for vegetable sales at markets in South Africa increased with 6.9% from 2010 to 2011 (DAFF, 2011). Thus it is safe to say that vegetable consumption is increasing. In 2011, South African markets sold on average 2 200 ton of lettuce at approximately R 3 500 per ton, 1 100 ton of green beans at approximately R 7 700 per ton, 23 000 ton of tomatoes at approximately R 4 500 and 9 500 ton cabbage at approximately R 1 400 per ton per month (DAFF, 2011). GCIS also reports that during 2009, 532 695 tons of tomatoes and 136 615 tons of cabbage were produced (GCIS, 2011). The loss of this amount of money would leave a big gap in the economy of South Africa and the world.

Farmers have various environmental factors and market risks counting against them (Vink & Van Rooyen, 2009). Thus in order for them to eliminate the risk of a great economic loss, it is of importance to ensure a safe product. Farm workers in South Africa have decreased extensively from 1970 to 2005; from approximately 1.6 million to 628 000 workers (Goldblatt, 2012). Unemployment is a reality in South Africa and agriculture is contributing to this since farms are becoming more mechanised (Vink & Van Rooyen, 2009). It is thus clear that if a foodborne outbreak linked to fresh produce occurs, it will result in increased unemployment as well. According to Hyslop (2011) a selection of retail outlets has detected *E. coli* on their fresh produce.

This indicates the difficulty for farmers to comply with health standards and thus it shows a problem for the food industry and to the economy.

CONCLUSION

Fresh vegetables are an important source of food for human consumption. Numerous rivers in South Africa are heavily polluted. Farmers tend to use river water for irrigation of vegetables and thus the risk of contamination increases.

McLellan (2004) and Power *et al.* (2005) indicated the presence of unique environmental strains of *E. coli*. Unique *E. coli* strains might also be present in the river water used for irrigation of fresh produce. It is therefore necessary to know which *E. coli* strains are present on fresh produce and of course how prevalent they are.

Throughout literature it is duly noted that there is a risk for the contamination of fresh vegetables as shown by the numerous international outbreaks caused by *E. coli*. The information available in South Africa, however, is limited in this regard. The little information available on the microbiological quality of South Africa's fresh produce does however indicate that foodborne pathogens might be present. Studies indicating the presence of *E. coli* have been conducted but the determination of the specific types of *E. coli* strains (phenotypes, genotypes, pathotypes) has not been done. It is therefore important to conduct such a study to determine the significance, or not, of the prevalence and types of *E. coli* on fresh vegetables in South Africa. Ultimately it is necessary to determine the risk involved concerning the presence of foodborne pathogens on fresh produce and also how to reduce, eliminate or prevent it. The effect of a foodborne pathogen surfacing will not only have a direct effect on the consumer and food industry but also on the economy in terms of unemployment.

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CHAPTER 3

PREVALENCE OF COLIFORMS AND *ESCHERICHIA COLI* ON FRESH PRODUCE FROM RETAIL STORES, FARMERS' MARKETS, SMALL-SCALE AND COMMERCIAL FARMS

SUMMARY

A total of 151 fresh produce samples were sourced from farms, farmers' markets and retail outlets. The produce samples consisted of lettuce, tomatoes, beans, peas, coriander, basil, mint, rocket, thyme, spinach, cabbage, parsley and sprouts. Total coliforms (TC) and *E. coli* loads on the produce were determined with Colilert-18. Isolates were phenotypically identified with the API Web system and the *E. coli* identification confirmed with *uidA* PCR.

The TC loads for the produce from the farms, farmers' markets and retail outlets were all in the same range from log 3 to log 8.4 MPN.100 mL⁻¹. *Escherichia coli* was found to be most prevalent on produce samples from farmers' markets with the highest *E. coli* load (log 7.38 MPN.100 mL⁻¹) on cabbage sampled from a commercial farm. *Escherichia coli* were found on 8% of the produce samples tested. Sixty-three isolates were identified as *E. coli* of which three were positive for the *uidA* gene but not identified as *E. coli* with the API Web system. The maximum TC and *E. coli* loads found on the fresh produce were log 8.38 MPN.100 mL⁻¹ and log 7.38 MPN.100 mL⁻¹, respectively. Data from the fresh produce sourced from retail outlets indicated the lowest risk in terms of TC and *E. coli* presence and load. Dendrograms and a PCA plot were statistically constructed with the Sokal and Michener (S_{SM}) and Jaccard (S_J) coefficients to determine similarity and dissimilarity groupings of the isolated strains and three main *E. coli* clusters were formed.

It was concluded that the type of *E. coli* present on a certain produce type or on produce from a certain source type could not be predicted. This could be an indication of a possible risk to the consumer if it is unknown on which produce and at which source specific pathogens are most likely to occur. This could also be a positive observation, as a specific contamination source could not be identified and thus the chance of localised disease outbreak is decreased.

INTRODUCTION

Fresh produce is generally considered healthy fresh and tasty (Hanif *et al.*, 2006). Various factors can contribute to produce being considered a hazard to the consumer. These can include contaminated irrigation water, unhygienic handling and polluted soil as sources of pathogens which can then be transferred to the fresh produce during the cultivation phases (Beuchat, 2002).

In South Africa agriculture is the largest user of surface water for irrigation (GCIS, 2011). South Africa is a water scarce country and sources other than municipal water, like river water, which is mainly used as irrigation water source. Many SA rivers have been reported to be unsuitable for irrigation purposes as a result of the high levels of faecal and microbial contamination (Barnes & Taylor, 2004; Gemmel & Schmidt, 2012; Britz *et al.*, 2013). These include the: Mosselbank; Berg; Plankenburg; Mhlathuze; Vuwanie; Mutshindudi; Tshinane; Mutale;

Mudaswali and Levubu Rivers (Bezuidenhout *et al.*, 2002; Obi *et al.*, 2002; Lin *et al.*, 2004; Paulse, *et al.*, 2012; Britz *et al.*, 2013). In many cases the faecal coliform levels exceed the World Health Organization (WHO) and Department of Water Affairs (previously known as Department of Water Affairs and Forestry = DWAF) guidelines for water to be used for the irrigation of fresh produce (WHO, 1989; DWAF, 1996). Different microbial types have been reported to be present in river water and have been shown to be carried over to crops during irrigation (Van Blommestein, 2012).

Escherichia coli can be either non-pathogenic or pathogenic and is present where faecal contamination occurs. There is, however, more than one type of pathogenic *E. coli* (Kaper *et al.*, 2004; Russo & Johnson, 2009; Carlos *et al.*, 2010). These pathogenic *E. coli* are a diverse group and every pathogenic type has a different mechanism of pathogenesis and can be resistant to different antibiotics (Lanz *et al.*, 2003; Reinthaler *et al.*, 2003; Kinge *et al.*, 2010).

Foodborne outbreaks caused by *E. coli* and linked to fresh produce are increasing (Lynch *et al.*, 2009; IUFOST, 2011). This is already resulting in consumers not trusting specific products and subsequently not purchasing them as fresh produce. It is thus of importance to ensure that fresh produce is safe to consume but this is only possible if action is taken to ensure that the microbial contamination on food products are reduced to acceptable levels according to the Health Protection Agency, the NSW Food Authority and the Department of Health guidelines (HPA, 2009; NSW Food Authority, 2009; DoH, 2011).

The objective of this research is to determine the prevalence of total coliforms and *E. coli* present on fresh produce sourced from farms, farmers' markets and retail stores in the Western Cape Province. The prevalence will be determined in terms of microbial loads and frequency present on the fresh produce from farms, farmers' markets and retailers. Additionally, colonies presumptively identified as *E. coli* will be further phenotypically characterised and the isolates grouped based on similarities. In conclusion, the risk linked to the prevalence of *E. coli* on the produce will be discussed.

MATERIAL AND METHODS

Samples and sampling sites

The fresh produce samples used in this project included a range of vegetables and herbs (lettuce, tomatoes, beans, peas, coriander, basil, mint, rocket, thyme, spinach, cabbage, parsley and sprouts) that are normally consumed raw by the consumer. The sampling sites included retail outlets, farmers' markets and farms. For this study, small-scale and commercial farms were categorised as point-of-harvest (PoH) samples, while retail outlets and the farmers' markets as post-harvest (PH) samples.

The retail outlets were sampled three times over a period of 18 months (approximately once every 6 months). The farmers' markets were sampled once or twice, depending on produce availability, over a 12 month period. The farms were sampled once every month for 6 months.

Due to seasonal aspects and availability limitations, not all samples were sampled and tested as often as was desired.

Farms (PoH samples)

The PoH samples were harvested directly on the farm. To protect the privacy of the farms they are labelled as Farm-1 to Farm-6. The farms used for the PoH sampling consisted of both large commercial and small-scale farms.

Farmers' markets (PH samples)

The farmers' markets were all in the Western Cape Province and represent a range from roadside stalls to organic markets. A broad spectrum of farmers' markets was sampled and these were labelled as Market-1 to Market-7.

Retail outlets (PH samples)

The samples from the retail outlets form part of the PH group and were all in the Stellenbosch region. From experience (D. February, Food Technologist, Pick 'n Pay, Personal communication, 2011) it was argued that retailers have the same produce suppliers, thus it did not matter where in the Western Cape the retail store is situated. The names of the retailers are not given so as to protect their privacy and samples recorded as Retail-1 to Retail-5.

Sampling methods

Point-of-harvest

The samples taken at the PoH sites were sampled directly at the farm in the crop fields. Sampling (300 - 500 g, in duplicate) was done aseptically, the samples placed in sterile plastic bags and then transported to the laboratory in insulated containers at 4°C and tested within 6 h.

Post-harvest

PH fresh produce samples (300 - 500 g, as pre-packed samples, in duplicate) were purchased from retail outlets. The same sampling strategy was not possible at the farmers' markets as the produce was mostly presented in open containers for the convenience of the consumer. For this study the farmers' market samples were placed in sterile plastic bags and transported at 4°C to the laboratory and tested within 6 h.

Microbial detection

Enumeration process

The removal of coliforms and *E. coli* present on the fresh produce surface during the enumeration process is the first step to determine the prevalence. In order to determine the optimum detection method, two methods were evaluated. The two options consisted of i) stomaching the fresh

produce sample, and ii) washing the surface of fresh produce samples in the sterile bags. Both were done in stomacher bags containing 400 mL sterile saline solution (SSS) (0.86% m/v). The reason for evaluating the two methods was that later in this study it was essential to obtain values for only the organisms present on the surface of the produce. If the samples are stomached the microbial load from both the surface and from the inside of the produce will be obtained.

For this part of the study 90 fresh produce samples were tested in duplicate for each method. As an example one sample set, e.g. from one packet of beans, 300 g beans was placed into two separate bags. Thus a total of 600 g beans was sampled. The sample size of 300 g per 400 mL SSS was used based on the same method used in a previous study by Van Blommestein (2012). After stomaching or washing, they were used for further analysis with the Colilert-18 method.

The results were statistically analysed with the ANOVA split plot method (Clewer & Scarisbrick, 2001). The null hypothesis was: counts from washed fresh produce equals counts from stomached fresh produce.

Prevalence determination

The Colilert-18 (IDEXX) method was used to determine the total coliforms (TC) and *E. coli* (EC) loads on the fresh produce samples according to the SANS 9308 method (SANS, 2012). A 300 g produce sample was placed in a stomacher bag and 400 mL SSS added. The stomacher bag with its contents was then carefully washed by hand for approximately 2 min. The washwater was then used to prepare a dilution series up to at least a 10^{-4} dilution. Each dilution was 100 mL in volume and this was then poured into a Quanti-Tray/2000 (IDEXX), adding the Colilert-18 reagent, sealed, and then incubated for 18 h at 35°C. A Quanti-Tray Sealer Model 2X (IDEXX) was used to seal the Quanti-Trays. The results were recorded after 18 h incubation by counting the wells that developed a yellow colour, indicating total coliforms, and then counting the wells that fluoresced under ultra violet light (365 nm), indicating the presence of *E. coli*. The number of positive wells for both total coliforms and *E. coli* were used to determine the corresponding loads from a table indicating the most probable number value (IDEXX; SANS, 2012).

Isolation of E. coli

The Colilert-18 Quanti-Trays method was used as base source to isolate *E. coli* present on the sampled produce. To do this the 48 large wells on the Quanti-Tray were divided into four blocks containing 12 wells each. After sterilising the outer surface of the wells, a maximum of two wells positive for *E. coli* from each block (1 mL per well) were all sampled and placed in a sterile container. This was then used to prepare a dilution series on Eosin Methylene Blue Agar (Levine) (L-EMB agar) (Oxoid) spread plates. A minimum of seven colonies from the plates with the best growth distribution were isolated using the Harrison Disk method (Harrigan & McCance, 1976). In simple terms, the Harrison Disk method is a method to select colonies in such a manner to obtain

isolates representing the entire plate. Each colony isolated from L-EMB plates was streaked at least twice on Brilliance™ *E. coli*/coliform selective medium (Chromogenic agar) (Oxoid) to ensure pure colonies (microscopically confirmed).

Phenotypic identification

The API 20E system (Biomérieux, South Africa) was used to identify the isolated organisms to species level. Streak plates were made on Nutrient Agar (NA) (Merck) to obtain single colonies. For this study all of the isolates were Gram stained and confirmed as Gram negative. A single colony was transferred to 5 mL SSS and homogenized and then used to fill the API 20E strip according to the manufacturers' instructions and incubated at 37°C for 18 – 24 h. Mobility was tested using the method of Doetsch (1981) and the OF (oxidative/fermentative) test was done using the method of Hugh & Leifson (1953). The API strip results were then entered into the API Web data system (Biomérieux, South Africa) to obtain the identification result for each strain tested.

Reference strains

Five ATCC (American Type Culture Collection) strains were included in this study: ATCC 13135 (R404); ATCC 10799 (R158); ATCC 4350 (R157); ATCC 11775 (R58); and ATCC 25922 (R25922). Twenty-two isolates from previous studies (Huisamen, 2012; Van Blommestein, 2012) were also incorporated into this study as marker isolates. These marker isolates were originally sampled from beans grown at two different sampling sites. The one sampling site was on a farm and the plants were hand irrigated and the other sample site was hand irrigated under more controlled conditions in a tunnel (Huisamen, 2012; Van Blommestein, 2012). The water used for the irrigation of both the above studies was from the Plankenburg River (site Plank-3). All 22 isolates (Huisamen, 2012; Van Blommestein, 2012) were included in the API 20E identifications.

Statistical clustering

Statistical clustering was done on the API 20E data obtained to configure similarity dendrograms, correspondence analysis and principal component analysis plots (XLStat 2011.1.01). For the numerical clustering the characteristics of 81 strains, including the 22 marker isolates from the Huisamen and Van Blommestein collection, isolated from L-EMB agar and purified on Chromogenic agar were included together with the five ATCC reference strains. The twenty-seven API characters were included in the data set and then analysed using the Jaccard (S_J) and Sokal and Michener (S_{SM}) coefficient methods. The unsorted similarity matrix was rearranged into groups by average linkage cluster analysis (Lockhart & Liston, 1970). Dendrogram distances were calculated based on the phenotypic characteristics as calculation concept (Personal communication, Ms N. Ntushelo, 10 April 2013, ARC Infruitec, Biometry Unit, Stellenbosch).

Isolate preservation

The isolates were grown on Chromogenic agar (Oxoid) and a single colony was transferred to 10 mL MRS broth (Merck) and incubated for 24 h at 37°C. A sample (500 µL) of the culture was added to 500 µL of sterile glycerol (80%) (Sigma-Aldrich, Malaysia) in a sterile cryotube (Greiner Bio-one, Germany) and then stored at -80°C.

Confirmation of isolate identity as *E. coli*

DNA template preparation

Isolates were cultivated on Tryptone Soya Agar (TSA) (Oxoid) at 37°C for 24 h. A colony was transferred to a 1.5 mL microcentrifuge tube (Quality Scientific Plastics®) containing 100 µL sterile nuclease-free water (SABAX Pour Water, Adcock Ingram Critical Care, South Africa), boiled for 13 min, cooled on ice and centrifuged at 14 000 x g for 15 min. The supernatant, containing the DNA template from the specific isolate, was transferred to a 0.6 µL sterile centrifuge tube and then stored at -18°C (Altafi & Hassen, 2009).

PCR - uidA gene amplification

The *uidA* gene was used to confirm the identity of an isolate as *E. coli* since this gene is a significantly preserved *E. coli* gene (Martins *et al.*, 1993; Feng & Lampel, 1994). The method of Heijnen & Medema (2006) was used on all of the *E. coli* strains isolated from fresh produce to confirm the identity as similar to that from API 20E results.

One PCR reaction is required to take place by amplifying the 187 bp *uidA* gene for each isolate. Each PCR tube contained 0.625 U KAPATaq HotStart DNA Polymerase, 0.5 µL of template DNA, 2.5 mM MgCl₂, 1X KAPATaq HotStart buffer, 0.8 mM dNTP mix and 0.4 µM of each primer UAL 2105b (5'-ATTGTTTGCCTCCCTGCTGC-3') and UAL 1939b (5'-ATGGAATTTGCGCGATTTTGC-3') (Heijnen & Medema, 2006) together with nuclease-free water to give a total reaction volume of 25 µL.

To verify the test, positive and negative controls were also added. The positive control contained a standard *E. coli* strain as template DNA (ATCC 11775) and the negative control contained nuclease-free water instead of template DNA.

All PCR reactions were performed in a G-Storm Thermal Cycler (Vacutec, South Africa). The reaction conditions were as follows: initial denaturation for 3 min at 95°C, 35 cycles of denaturation for 30 s at 95°C, primer annealing for 30 s at 59.7°C, extension for 30 s at 72°C, followed by a final extension for 5 min at 72°C.

Gel electrophoresis was then done to view the PCR products. A 1% agarose (SeeKem) gel containing 1 µg.mL⁻¹ ethidium bromide (Sigma) was used. The gel electrophoresis was carried out with a Baygene-BG-Power300 power supply (Vacutec, South Africa) for 20 min at 210 V. The bands were visualised on a UV transilluminator (Vacutec, South Africa).

RESULTS AND DISCUSSION

Enumeration

At the start of the study two methods were evaluated for enumeration of the total coliforms (TC) and *E. coli* in order to determine which one of the washed fresh produce or stomached fresh produce would provide the highest counts and lowest variation. The data obtained was statistically analysed using the ANOVA split plot (Clewer & Scarisbrick, 2001). The ANOVA results are given in Table 1.

Table 1 ANOVA Split plot of data for the two enumeration methods evaluated

Source of variance	Df	SS	MS	F-ratio
Treatments	1	2.55241×10^{15}	2.55241×10^{15}	3.729
Error	178	1.2181059×10^{17}	6.843292×10^{14}	
Total	179	1.24363×10^{17}		

Df = degrees of freedom; SS = sum of squares; MS = mean square; F-ratio = variance ratio

The null hypothesis, counts from washed fresh produce equals counts from stomached fresh produce, was rejected; $F_{1;178;0.05} = 3.729$ (Table 1). Overall the microbial results from the produce sampled with the two methods did not differ with the exception of the tomatoes. The tomatoes that were washed gave higher loads ($\log 5.38 \text{ MPN.100 mL}^{-1}$) compared to the stomached tomatoes that gave very low counts ($\log 3.92 \text{ MPN.100 mL}^{-1}$). The tomatoes were completely crushed when stomached and thus the result was not considered as accurate as it was found that the red pulp interfered with the reagents used in the Colilert-18 detection method that includes the sealing of a tray and a non-typical colour reaction. The variance in the results from the washed samples were lower than the variance from the results from the stomached samples (Table 1). The standard deviation ($\log 6.84 \text{ MPN.100 mL}^{-1}$) from the washed samples' data was also lower than the standard deviation ($\log 7.56 \text{ MPN.100 mL}^{-1}$) from the stomached samples' data. It was thus concluded that the washing of produce instead of stomaching resulted in a more optimal enumeration.

The reason for using the washing method can be explained in that it presents more stable results due to the variance and standard deviation being smaller for the washed samples than for the stomached results. Additionally, the fresh tomato produce samples prepared with the stomacher method resulted in detection problems as a result of the colour, gelling and pH changes making the data difficult to read when using the Colilert-18 method.

The objective of the project was also to determine the prevalence of coliforms and *E. coli* on the surface of fresh produce as this project was ultimately linked to a larger WRC project

evaluating the linking of contaminated irrigation water to the presence of faecal contaminants on the surface of the fresh produce. This study was part of an on-going solicited research project (K5/1773) funded by the Water Research Commission and co-funded with the Department of Agriculture, Forestry and Fisheries (DAFF). Thus, when taking all of the fresh produce types into consideration, it was concluded that the produce washing method resulted in higher and more reproducible results.

Total coliforms (TC) and *E. coli* on fresh produce at point-of-harvest and post-harvest

The combined TC (Total coliforms) and *E. coli* data from the farms, farmers' markets and the retail outlets (Fig. 1), shows the levels of TC and *E. coli* present on the fresh produce samples.

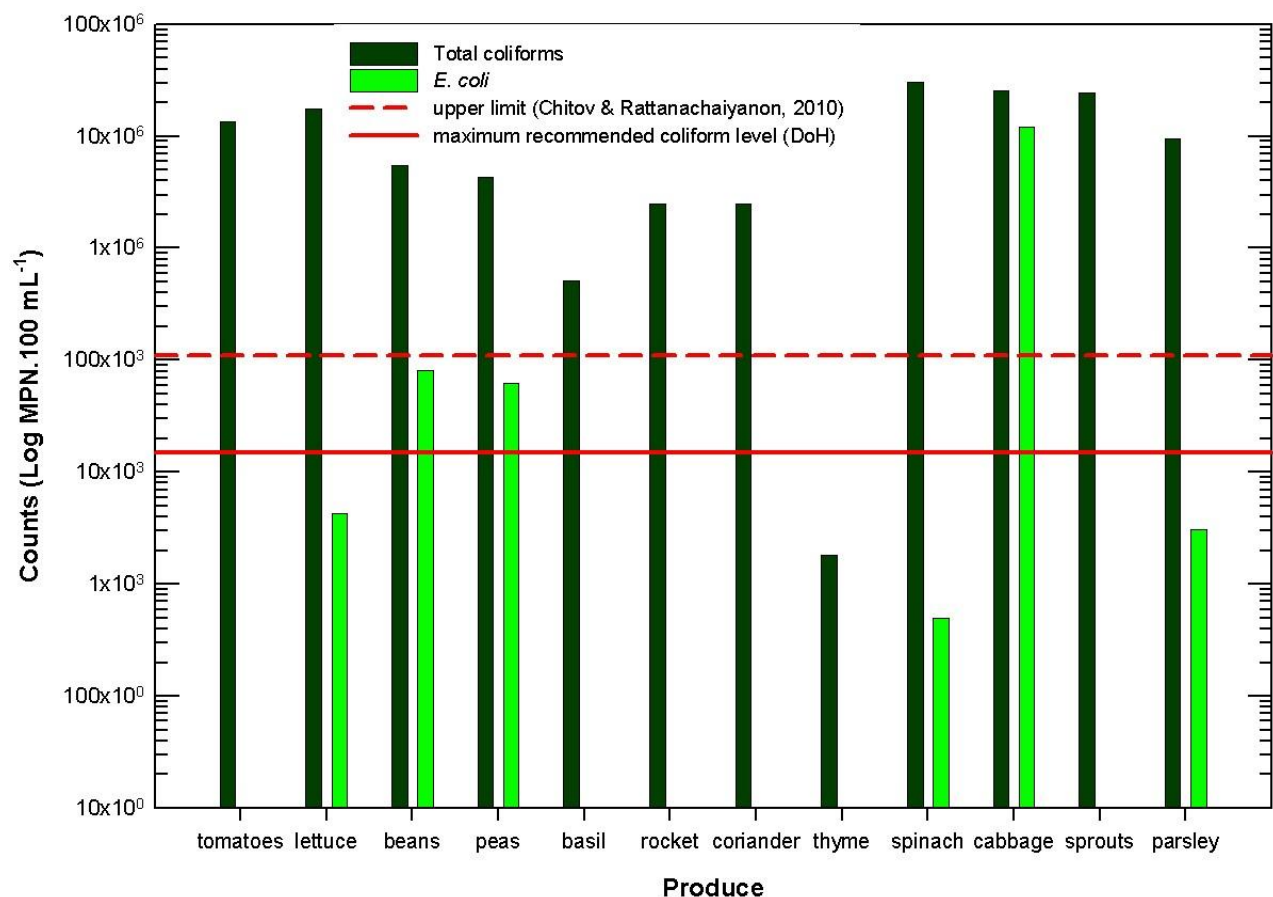


Figure 1 Average levels of TC and *E. coli* on fresh produce. The solid horizontal red line on the bar graph indicates the maximum recommended level for coliforms on raw vegetables ($<200 \text{ cfu.g}^{-1} = <15 \text{ 000 cfu.100 mL}^{-1}$) (DoH, 2011). The dotted red line on the bar graph indicates the upper limit as the maximum recommended level for coliforms on raw vegetables ($<110 \text{ 000 MPN.100 mL}^{-1}$) (Chitov & Rattanachaiyanon, 2010). (The dark green bars represent the TC and the light green bars represent the *E. coli*).

As indicated in Fig. 1, the maximum recommended level for TC on raw vegetables is $<200 \text{ cfu.g}^{-1}$ ($<15\,000 \text{ cfu.100mL}^{-1}$) (DoH, 2011). The maximum recommended level set by the South African Department of Health for *E. coli* on raw vegetables is 0 cfu.g^{-1} ($0 \text{ cfu.100 mL}^{-1}$) (DoH, 2011). In order to compare the results from this study (given as MPN.100 mL⁻¹) to the recommended maximum TC level of the South African Department of Health (given as cfu.g^{-1}) (DoH, 2011), the following assumptions calculation were made: cfu.g^{-1} counts were converted to cfu.mL^{-1} counts according to the original sample to the diluent ratio used in this study (given as 300 g in 400 mL), therefore 200 cfu.g^{-1} as recommended by the South African Department of Health (multiply by 300 (g) and then divided by 400 (mL)) would thus be 150 cfu.mL^{-1} or $15\,000 \text{ cfu.100 mL}^{-1}$. Then to convert the cfu value to a MPN value, the value of $15\,000 \text{ cfu.100 mL}^{-1}$ was firstly converted directly to $15\,000 \text{ MPN.100 mL}^{-1}$ based on the “assumption” that the results obtained using the two methods, are identical. For this reason, $<15\,000 \text{ cfu.100 mL}^{-1}$ was shown in Fig. 1 as the DoH recommended guideline (2011).

However, since Cho *et al.* (2010) concluded that enumerated *E. coli* using the MPN method is in most cases greater than the enumeration using the cfu method for the same sample, it was considered necessary to also include an upper limit for this study. For this purpose the upper limit of the cfu-to-MPN conversion table of Chitov & Rattanachaiyanon (2010) determined for *E. coli* in pure culture and on raw food was included in Fig. 1. This upper limit was calculated as follows: $101 - 500 \text{ cfu.mL}^{-1} = 1\,100 \text{ MPN.mL}^{-1}$ or $110\,000 \text{ MPN.100 mL}^{-1}$ (Chitov & Rattanachaiyanon; 2010).

It is clear from the results and lines shown in Fig. 1 that in most cases the TC levels exceeded the DoH maximum recommended guideline for the different types of produce. The exception was thyme that had TC loads below the recommended guidelines.

In the case of *E. coli* it was found to be present on only six of the 12 produce types sampled (Fig. 1). However the recommended maximum level for *E. coli* was exceeded in all cases (recommended maximum level is 0 cfu.g^{-1} ($0 \text{ cfu.100 mL}^{-1}$) (DoH, 2011)). Overall, the levels of *E. coli* present were found to be generally lower than the TC levels.

The total coliforms belong to the family *Enterobacteriaceae* and have been defined in the 20th edition of Standard Methods for the Examination of Water and Wastewater (APHA, 1998). The family *Enterobacteriaceae* (Brenner *et al.*, 2005) presently consists of at least 42 different genera with similar characteristics. Some of the genera are of faecal origin but others are found exclusively in the environment. Coliforms are generally used as indicators of possible faecal contamination and are of sanitary significance. Although not all genera originate from the intestinal tracts of humans and animals they can dominate in the water environment. Even though there are short comings in using the “total coliform” group as an indicator, they do internationally form part of the primary standards for potable water (Hurst *et al.*, 2007). Coliforms consist of a group of bacteria that include the *Klebsiella*, *Salmonella*, *Shigella*, *Serratia*, *Hafnia*, *Citrobacter*, *Escherichia* and *Enterobacter* species (Brenner *et al.*, 2005) and thus the presence of TC in water or foods can

be an indication of the presence of any of the bacteria species classified as TC and not only *E. coli*. It was also clear from the data of this study (Fig. 1) that TC is not really a good indicator of faecal contamination or even as an indicator of the presence of *E. coli*. This can be explained when studying Figs. 1 and 2 where the average TC loads detected on the spinach samples ($\log 7.5 \text{ MPN} \cdot 100 \text{ mL}^{-1}$) were much higher than the *E. coli* loads ($\log 3.7 \text{ MPN} \cdot 100 \text{ mL}^{-1}$) compared to the average *E. coli* loads on the cabbage samples ($\log 7.08 \text{ MPN} \cdot 100 \text{ mL}^{-1}$) which are not much less than the TC loads ($\log 7.50 \text{ MPN} \cdot 100 \text{ mL}^{-1}$) detected on the cabbage.

The presence of *E. coli* on produce is taken as an indication of faecal contamination as *E. coli* is known as a common inhabitant of warm blooded animals' intestinal tract (WHO, 1996) and thus can be taken as an indication of risk to the consumer. Based on the data from this study it might not be a serious risk in terms of the level of counts present on the produce that the consumer will purchase but it still gives an indication of the possibility of an unsafe produce product.

Farms (PoH) - small-scale and commercial farms

The TC data (Fig. 2) collected from produce sampled at small-scale and commercial farms showed a large variation in loads varying from a minimum of $\log 3$ to a maximum of $\log 8.38 \text{ MPN} \cdot 100 \text{ mL}^{-1}$. The variation in the prevalence of TC on the fresh produce sampled from six farms is clearly indicated in Fig. 2. The horizontal lines in the green bars indicate the average loads. The cabbage had the highest load ($\log 8.38 \text{ MPN} \cdot 100 \text{ mL}^{-1}$), and this one sample can be seen as an outlier in Fig. 2. Parsley had the highest average load at $\log 6.98 \text{ MPN} \cdot 100 \text{ mL}^{-1}$. The range of TC detected was the highest on cabbage and thus it can be presumed to be a higher risk for pathogen presence.

In the case of *E. coli*, they were only detected on one of the parsley samples at a level of $\log 3.49 \text{ MPN} \cdot 100 \text{ mL}^{-1}$. In contrast, Thomas *et al.* (2009) reported data indicating the presence of pathogenic *E. coli* on tomatoes and cabbages even when the TC counts were low and higher TC counts when no pathogenic *E. coli* was present. All of the TC results for the farm samples (PoH) exceed the recommended guidelines for raw vegetables set by the Department of Health (DoH, 2011) ($<200 \text{ cfu} \cdot \text{g}^{-1} = <15\,000 \text{ cfu} \cdot 100 \text{ mL}^{-1}$) for TC. Mukherjee *et al.* (2004) reported coliform counts on tomatoes, leafy greens, lettuce and cabbage from farms in Minnesota, as 2.3, 3.3, 4.0, and 2.6 $\log \text{ MPN} \cdot \text{g}^{-1}$, respectively. These values are similar to the data from this study with the higher counts found on cabbage and lettuce and lower counts found on tomatoes (Fig. 2).

In total, eight cabbage samples were tested and *E. coli* only detected on two samples. Only one parsley sample showed the presence of *E. coli*. *Escherichia coli* was only detected on cabbage and parsley samples sourced from farms. The high TC loads from this sampling source did give an indication of the presence of *E. coli* but this is not always the case, especially with pathogenic *E. coli* as previously mentioned (Thomas *et al.*, 2009). The *E. coli* on the cabbage ranged from $\log 3.61$ to $\log 7.38 \text{ MPN} \cdot 100 \text{ mL}^{-1}$ while the load on parsley was $\log 3.49 \text{ MPN} \cdot 100$

mL⁻¹. These loads also exceeded the DoH recommended guidelines <0 cfu.g⁻¹ (<0 cfu.100 mL⁻¹) for the presence of *E. coli* on raw vegetables (DoH, 2011).

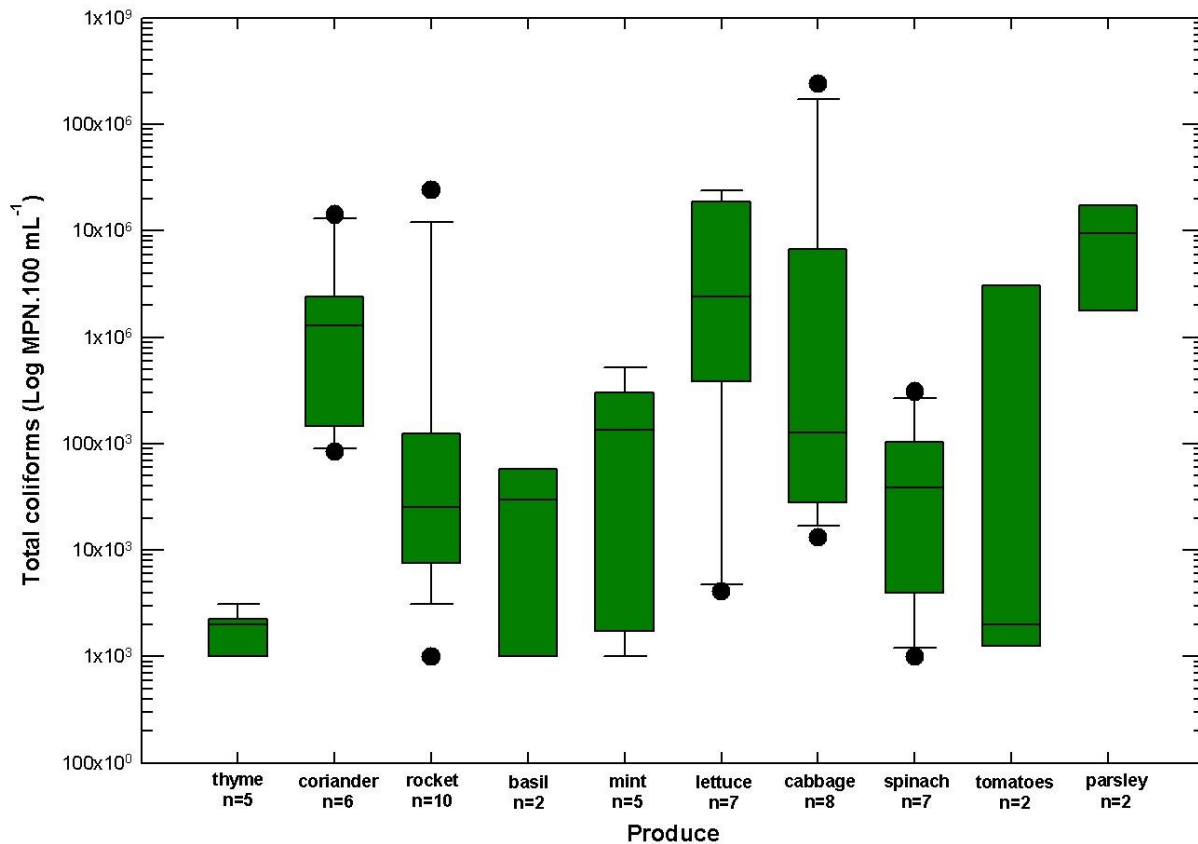


Figure 2 Total coliforms loads present on fresh produce from farms. The black dots represent values that are extreme outliers. The error bars indicate the 90th and 10th percentiles above and below the box where applicable, respectively. The absence of error bars indicate that the range of results detected was not large enough to indicate an error. The horizontal line inside the green bar indicates the average value for the specific produce type. The green bar indicates the load range present.

Initially two cabbage samples were sourced on the same day so as to determine the load variation between samples from the same day. On the one cabbage sample log 4.40 MPN.100 mL⁻¹ TC was detected (*E. coli* absent) and on the second sample log 8.38 MPN.100 mL⁻¹ TC with log 8.38 MPN.100 mL⁻¹ *E. coli* was found. Various factors can be the reason for the large difference in loads detected including unhygienic handling, contaminated transport containers, animals and harvest equipment. Since six out of the eight cabbage samples did not show any indication of *E. coli* with the exception of the one cabbage sample with log 8.38 MPN.100 mL⁻¹ *E. coli* present, the most evident cause could have been birds' excrement as the presence of

excrement was visible on the second cabbage sample. Based on the large microbial level variation it is recommended that farmers should ensure in some way that this variation be eliminated by pre-washing after harvest or before distribution.

Farmers' markets

Fresh produce samples from farmers' markets were also monitored for the presence of TC and *E. coli* and the TC loads summarised in Fig. 3.

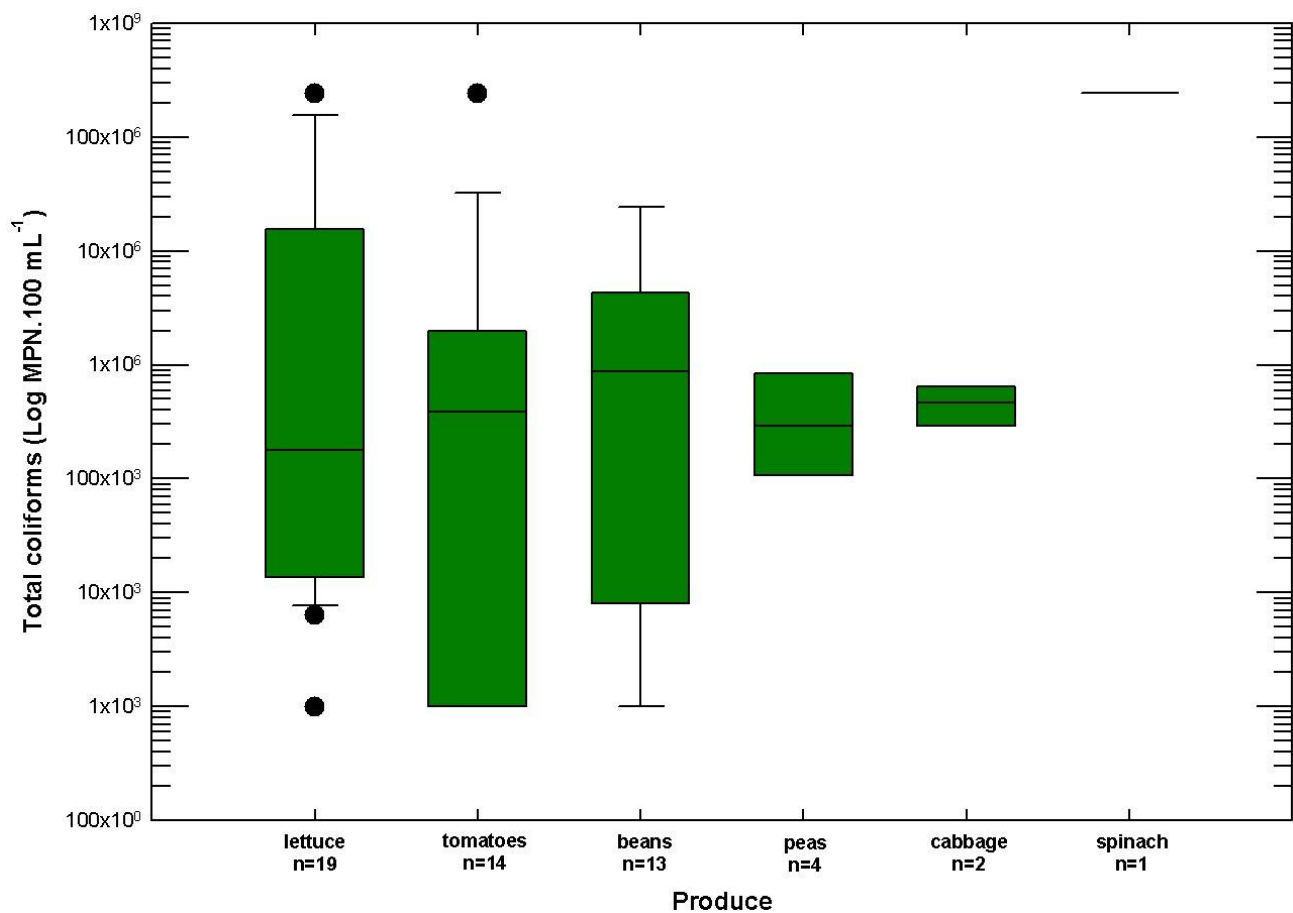


Figure 3 Prevalence of total coliforms on fresh produce from farmers' markets. The black dots represent values that are extreme outliers. The error bars indicate the 90th and 10th percentiles above and below the box, respectively. Where applicable the absence of error bars indicate that the range of results detected was not large enough to indicate an error. The horizontal line in the green bar indicates the average value for the specific produce type and the single horizontal line present for the spinach samples indicates the presence of TC on only one of the spinach samples. The green bar indicates the load range present.

The fresh produce samples from the farmers' markets included four farmers' markets and three organic markets. All the TC loads detected on this group of samples exceeded the recommended guidelines ($<200 \text{ cfu.g}^{-1} = <15\,000 \text{ cfu.100 mL}^{-1}$) for raw vegetables (DoH, 2011). In Fig. 3, it can be seen that the highest TC loads were on both the lettuce and tomatoes at log 8.38 MPN.100 mL⁻¹. The produce type that gave the highest average at log 6.67 MPN.100 mL⁻¹, was the beans. Only one spinach sample was tested due to lack of availability, but did show a high TC load at log 8.38 MPN.100 mL⁻¹.

Escherichia coli were detected on lettuce, beans, spinach and peas from the farmers' markets but not present on any of the other produce. *Escherichia coli* were only detected on one sample each of lettuce, beans and spinach. The loads were recorded as log 3.93 MPN.100 mL⁻¹ on lettuce, log 3.49 MPN.100 mL⁻¹ on beans and log 3.0 MPN.100 mL⁻¹ on spinach. Log 4.98 MPN.100 mL⁻¹ was the highest *E. coli* load and found on peas. All the *E. coli* loads detected exceeded the recommended guideline of 0 cfu.100 mL⁻¹ (DoH, 2011). A study done in Brazil reported *E. coli* to be present on 41% of produce samples from a farmers' market (Maffei *et al.*, 2013). This is a much higher percentage when compared to this study where *E. coli* was only found present on 11% (6 out of 53 samples) of the produce sampled from farmers' markets.

It is important to take into consideration that produce from farmers' markets is not pre-packed and is handled more by the consumer. It might, in some cases be fresher than produce in retail stores. When taking the above into consideration it is clear that produce (farmers' markets) could possibly be more contaminated than pre-packed produce. In the literature it has been reported that *E. coli* counts do decrease significantly after a week of being present on produce (Monaghan & Hutchison, 2008). In contrast it has been reported that *E. coli* O157:H7 was significantly present on injured lettuce leaves compared with non-injured lettuce leaves (Aruscavage *et al.*, 2008). This presence of high TC and *E. coli* loads on the fresh produce sourced from farmers' markets is similar to that found in this study.

Retail outlets (PH)

In this section produce samples sourced from five retail outlets were tested for the presence of TC and *E. coli*. The data obtained for the TC is presented in Fig. 4 and gives an indication of the average loads for each produce type and also the maximum and minimum values obtained.

The highest TC load found on the beans, lettuce, tomatoes, sprouts and peas from retail stores was log 7.38 MPN.100 mL⁻¹. As a result of seasonal variation and lack of availability during this study, only one sample of sprouts was tested and the TC load found was log 7.38 MPN.100 mL⁻¹. In all cases the TC loads on the produce exceeded the recommended TC guidelines for raw vegetables $<200 \text{ cfu.g}^{-1}$ ($<15\,000 \text{ cfu.100 mL}^{-1}$) (DoH, 2011).

Escherichia coli was only detected on two bean samples at loads of log 2 MPN.100 mL⁻¹ and log 5.38 MPN.100 mL⁻¹ and exceeded the recommended guideline of $<0 \text{ cfu.g}^{-1}$ ($<0 \text{ cfu.100 mL}^{-1}$) (DoH, 2011). In other studies, Johannessen *et al.* (2002), found thermotolerant coliform

bacteria present on 5 out of 200 lettuce samples, 3 out of 130 herb samples and 5 out of 100 parsley samples, all sourced from retail stores and markets in Norway. *Escherichia coli* was not present on any of their produce samples. For this study, the results obtained for the retail stores' fresh produce samples (43 samples) were the lowest when compared to the loads on farms and farmers' markets samples and thus it was concluded that the TC and *E. coli* were the least prevalent at retail stores.

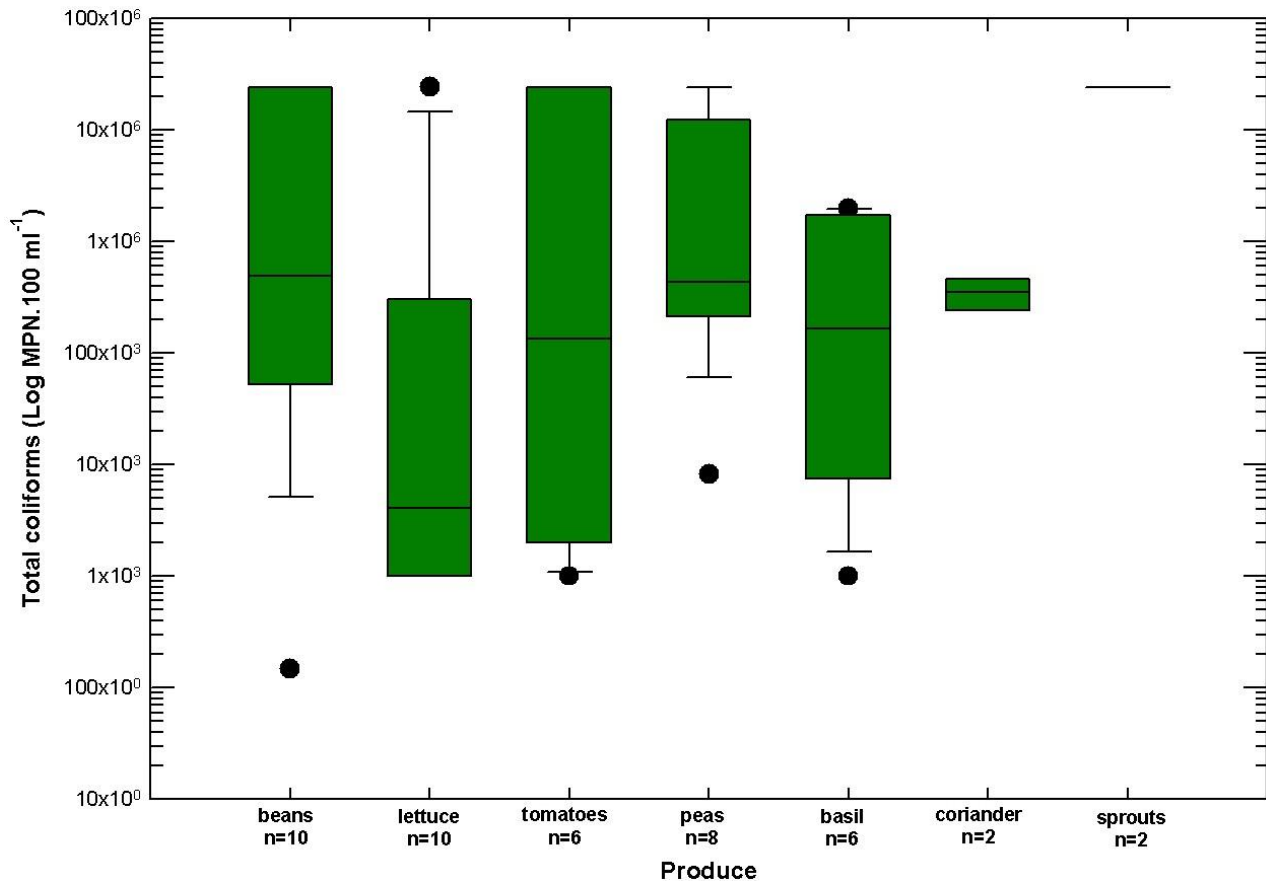


Figure 4 Prevalence of total coliforms on fresh produce from retail stores. The black dots represent values that are an extreme outlier compared to the other data points. The error bars indicate the 90th and 10th percentiles above and below the box, respectively. Where applicable, the absence of error bars simply indicate that the range of results detected was not large enough to indicate an error. The horizontal line in the green bar indicates the average value for the specific produce type and the single horizontal line present for sprouts indicates the presence of TC only present on one of two sprout samples. The green bar indicates the load range present.

Potential risk

A United Kingdom (UK) microbiological guideline for ready-to-eat foods was set out by Gilbert *et al.* in 2000. *Enterobacteriaceae* was the basic unit used in place of TC. In terms of the *Enterobacteriaceae* load it was regarded as satisfactory when $<100 \text{ cfu.g}^{-1}$ ready-to-eat food is detected, acceptable when 100 to $<10^4 \text{ cfu.g}^{-1}$ ready-to-eat food is detected and unsatisfactory when $\geq 10^4 \text{ cfu.g}^{-1}$ ready-to-eat food is detected. In terms of *E. coli* loads it was regarded as satisfactory when $<20 \text{ cfu.g}^{-1}$ ready-to-eat food were present, acceptable when 20 to $<100 \text{ cfu.g}^{-1}$ ready-to-eat food and unsatisfactory when $\geq 100 \text{ cfu.g}^{-1}$ ready-to-eat food were present. They also recommended that a separate evaluation should be done for *E. coli* O157 and other VTEC strains.

Thus these strains can either be present in a 25 g ready-to-eat food sample or absent in the food sample. When absent it was regarded as satisfactory and when present it was regarded as unacceptable/potentially hazardous (Gilbert *et al.*, 2000).

The Health Protection Agency (HPA) issued guidelines also to be used for the UK (HPA, 2009). The HPA also test for *Enterobacteriaceae* instead of TC. When testing for *Enterobacteriaceae*, it was regarded as satisfactory when $<10^2 \text{ cfu.g}^{-1}$ ready-to-eat food, borderline when 10^2 to $\leq 10^4 \text{ cfu.g}^{-1}$ ready-to-eat food and unsatisfactory when $>10^4 \text{ cfu.g}^{-1}$ ready-to-eat food were found. Borderline results suggest that the results be interpreted in conjunction with test results from other microbiological parameters but detection in several foods or other areas of the food production environment should be investigated and at unsatisfactory levels suggest that their presence at these levels indicate an overall poor general hygiene status of a food product (HPA, 2009). When testing for *E. coli*, they regarded it as satisfactory when $<20 \text{ cfu.g}^{-1}$ ready-to-eat food is detected, borderline when 20 to $\leq 10^2 \text{ cfu.g}^{-1}$ ready-to-eat food is detected and unsatisfactory when $>10^2 \text{ cfu.g}^{-1}$ ready-to-eat food were present (HPA, 2009). Borderline suggests that although *E. coli* should not be detected in ready-to-eat foods, low levels may occasionally be found. Repeated or widespread detection in several foods or areas of the food production environment suggests an increased food safety risk. Unsatisfactory results suggest that the detection of *E. coli* can signify a risk that faecal pathogens are present and that repeated or widespread detection in several foods or environmental sites highlights an increased food safety risk. When testing for *E. coli* O157 (and other verocytotoxin-producing *E. coli* (VTEC) according to the HPA, 25 g of ready-to-eat food is tested for presence or absence and thus the result will be regarded as detected or not detected. Detected is an indication of a high risk and thus unsatisfactory which is interpreted as potent (HPA, 2009). Not detected indicates a low risk and thus satisfactory.

The NSW Food Authority in their microbiological guidelines for Australia and New Zealand, interprets microbiological results in four result categories; good and acceptable, both regarded as pass, and fail as either unsatisfactory or potentially hazardous. Good is interpreted as results that are within expected microbiological levels for this type of product (lower range) and present no food safety concern (NSW Food Authority, 2009). Acceptable is interpreted as results that are within expected microbiological levels for this type of product (upper range) and present no food

safety concern. Unsatisfactory is interpreted as results that are outside the expected microbiological levels for this type of product, present no food safety concern, but might indicate poor food handling practices. Potentially hazardous is interpreted as results that are outside of the expected microbiological levels for this type of product and present a potential food safety concern.

The NSW Food Authority also tests for *Enterobacteriaceae* instead of TC. For *Enterobacteriaceae* a result of $<10^2$ cfu.g⁻¹ ready-to-eat food is good, 10^2 to $<10^4$ cfu.g⁻¹ ready-to-eat food is acceptable and $\geq 10^4$ cfu.g⁻¹ ready-to-eat food is unsatisfactory (NSW Food Authority, 2009). When testing for *E. coli* <3 cfu.g⁻¹ ready-to-eat food is good, 3 to $<10^2$ cfu.g⁻¹ ready-to-eat food is acceptable and $\geq 10^2$ cfu.g⁻¹ ready-to-eat food is unsatisfactory (NSW Food Authority, 2009).

When comparing the three guidelines discussed above to the DoH recommended guidelines (DoH, 2011) it is interesting to note that they (Gilbert *et al.*, 2000; HPA, 2009) regard it as acceptable when *E. coli* is detected at low levels where as DoH indicates that <0 cfu.g⁻¹ (<0 cfu.100 mL⁻¹) should be present. The levels that are regarded as acceptable by the UK and Australia and New Zealand are still low and the UK guidelines (Gilbert *et al.*, 2000; HPA, 2009) do indicate that if pathogenic *E. coli* (specifically *E. coli* O157 and other VTEC) are present it is regarded as unsatisfactory or unacceptable/potentially hazardous (Gilbert *et al.*, 2000; HPA, 2009).

When the NSW and UK guidelines are applied to the data obtained in this study it can be argued that the prevalence of *E. coli* as found on produce is low indicating that the probability of a pathogenic *E. coli* strain being present is low and thus not a big concern. According to the DoH recommended guidelines, however, *E. coli* should be completely absent otherwise it can be seen as a risk.

A hazard is defined as an agent having the potential to cause a health risk and a risk can be defined as the probability of a hazard causing a negative health effect and the extent thereof (Griffith, 2012). *Escherichia coli* can be both beneficial to the human body by enhancing function in the intestines or it can be harmful as a pathogen. As a result the presence of *E. coli* does qualify as a health hazard (Griffith, 2012) as it is able to acquire genes from other *E. coli* strains. Pathogenic *E. coli* can be grouped further into two groups, intestinal pathogenic *E. coli* and extraintestinal pathogenic *E. coli*, and then even further into six (EHEC, EAEC, ETEC, EIEC, EPEC, DAEC) and three (UPEC, SEPEC, NEMEC), respectively (Kaper *et al.*, 2004; Russo & Johnson, 2009). Each of these pathotypes has a different method of pathogenesis (Percival *et al.*, 2004; Bhunia, 2008). Pathogenic *E. coli* can either produce heat stable toxins or be pathogenic their mode of action (Percival *et al.*, 2004; Bhunia, 2008). Thus, the fact that there is more than one type of pathogen to be aware of makes the presence of *E. coli* on a foodstuff an even bigger risk. It can thus be further argued that it is a risk to the consumer when *E. coli* was present on the produce as this also indicates the possibility of it occurring again. Throughout harvest, processing and packaging there is no killing step as the fresh produce is consumed raw. Due to this it is essential to ensure that the final raw product for the consumer is free from pathogenic bacteria.

For this study a risk is defined as the probability of a negative health effect or hazard and the extent thereof. It can further be described as a qualitative or quantitative risk. In effect, the data from the farms, retail outlets and mostly farmers' markets indicated faecal contamination. In this case it is a qualitative risk as the data are insufficient to estimate the risk using an equation. The TC loads present on produce from farms, farmers' markets and retail outlets are all in the same range from approximately $\log 3 \text{ MPN} \cdot 100 \text{ mL}^{-1}$ up to $\log 8.4 \text{ MPN} \cdot 100 \text{ mL}^{-1}$. *Escherichia coli* were the most prevalent on produce from farmers' markets and the least prevalent on produce from retail outlets. The data from the farmers' markets also indicates that the *E. coli* was present on more sample types and at higher loads. It was thus concluded that the retail outlets had the lowest risk in terms of fresh produce that will be consumed raw.

Since *E. coli* was detected on produce samples from farms, farmers' markets and retail outlets and thus it can be argued that all the sources could pose a potential health risk to the consumer. In a study done in the Eastern Cape on cooked vegetables no *E. coli* were detected but *Listeria* spp., *Enterobacter* spp. and *Staphylococcus aureus* among others, were detected (Nyenje *et al.*, 2012). This could have been due to post-processing contamination. Taking the data into consideration and the recommended guidelines for *E. coli* on raw produce (<0 CFU/g; <0 CFU/100 mL) (DoH, 2011), it can be speculated that there is an indication of risk. As *E. coli* should be absent on any raw vegetable product, it is regarded as a risk if any level of *E. coli* is detected on fresh produce.

Identification of *E. coli* strains isolated from fresh produce

Identification - API 20E

A total of 81 isolates were obtained from fresh produce samples from all the source types. These included 22 marker strains from previous studies (Huisamen, 2012; Van Blommestein, 2012), and subsequently identified with the API Web system as *E. coli*. Of the 81 isolates, 60 (including the 22 marker strains) were identified as *E. coli* type 1 and all had an identification percentage of 95 or higher. The API 20E profile and percentage identification for each isolate is given in Addendum A. The 18 strains that were not identified as *E. coli* were found to be members of either the genera *Raoultella* (1 isolate), *Enterobacter* (4 isolates), *Klebsiella* (5 isolates), *Kluyvera* (1 isolate), *Serratia* (1 isolate), *Pantoea* (3 isolates) or could not be identified with API (3 isolates).

The number of isolates identified as *E. coli* from each source location was as follows: one from retail stores, 23 from farmers' markets and 36 from farms. The 22 marker strains were all sourced from beans that had been hand irrigated with water from the Plankenburg River (Plank-3) and all identified as *E. coli* with a percentage identification of higher than 96. Five *E. coli* ATCC reference strains (ATCC 13135 = R404; ATCC 10799 = R158; ATCC 4350 = R157; ATCC 11775 = R58; ATCC 25922 = R25) were additionally included in the study. In total, the study included 68 *E. coli* strains.

Identification confirmation – uidA PCR

All the isolates, markers and the reference strains were evaluated for the presence of the *uidA* gene with PCR. The *uidA* gene is considered a highly conserved gene for *E. coli* (Martins *et al.*, 1993). All the API identified *E. coli* strains (Addendum A) were found to be positive for the *uidA* gene. Three other strains (Addendum A) tested positive for the presence of the *uidA* gene but these isolates showed a low discrimination and were not positively identified as *E. coli* with the API Web system. These three isolates (7, 15 and 18) attained percentage identification levels with the API Web system of less than 80%.

Statistical clustering

For the numerical clustering the characteristics of the 81 isolates (including the 22 marker strains from the Huisamen and Van Blommestein collections) from fresh produce and five the ATCC reference strains, were included. Twenty-seven API phenotypic characters were included in the data set and analysed using the Jaccard (S_J) and Sokal and Michener (S_{SM}) coefficients and the unsorted similarity matrix was rearranged into groups by average linkage cluster analysis (Lockhart & Liston, 1970). Dendrogram distances were calculated based on the phenotypic characteristics as calculation concept.

The S_{SM} takes into consideration all the reaction results, including positive, negative and the positive/negative variations. The S_J only takes into consideration the positive reaction results. Thus these two methods give different sets of clusters as each combination will differ.

All the isolates (Addendum A) from the study were included in constructing the S_{SM} dendrogram in Fig. 8. Nine clusters were identified based on dissimilarities. The clusters are numbered in squares on the dendrogram from A to I.

Clusters A, B, D, E and F all contain isolates that were not identified as *E. coli*. Of the nine clusters there were only four clear *E. coli* groups present (C, G, H and I). The variance decomposition (XLStat 2011.1.01) within the clusters was calculated as 14.39% with the S_{SM} coefficient constructed according to the method of Lockhart & Liston (1970). The variance decomposition simply gives an indication as to what degree the isolates within a cluster will diverge from each other.

In cluster C, all the isolates, with the exception of 50 and 7, were clearly identified as *E. coli* with the API 20E system. Isolate 7 did however test positive for the presence of the *uidA* gene with PCR, and was then accepted as *E. coli*. Isolate 50 was negative for the *uidA* gene and thus it was not identified as *E. coli*. Isolates 69, 73 and 81 had the same characteristics and isolate 7 only differed with two characteristics, SOR (sorbitol) and SAC (saccharose) which were negative and positive, respectively. Since isolate 7 is clustered in a group with other positively identified *E. coli* strains it shows that it does have similar characteristics as the other *E. coli* identified isolates in the cluster and was thus taken as further confirmation of the PCR results. All of the isolates in cluster

C were isolated from beans. This could be an indicator that *E. coli* strains with specific characteristics are prone to be found on beans.

The blue numbered isolates (80, 82, 63, 78, 76, 75, 74, 71, 70, 68, 67, 66, 61, 62, 64 and 65) in cluster G were all sourced from beans irrigated from the same river (Plankenburg River - site-3). These isolates are all closely related in terms of their phenotypic characteristics as shown by the small dissimilarity values in Fig. 8. This could be an indication that the *E. coli* strains that were isolated from the beans, could have originated from the river and were transferred via irrigation.

The strains in Cluster H had a wider phenotypic variation range. Isolates 15 and 18 were not identified as *E. coli* with the API Web system but they did test positive for the presence of the *uidA* gene with PCR and were thus considered as *E. coli*. Compared to isolate 16, identified as *E. coli* with the API, isolates 15 and 18 only differed with one characteristic being mobility negative. When compared to isolate 72, identified as *E. coli* with API, isolates 15 and 18 differed in terms of MOB (mobility) and ADH (L-arginine). It can be argued that because they are clustered with *E. coli* isolates, they have similar phenotypic characteristics and it thus again supports the positive *uidA* PCR characteristics.

The isolates in cluster H were all sourced from farmers' markets. Again, a similar strain or type of strain can possibly be present on produce from farmers' markets and thus it could additionally be argued that these sources may have similar irrigation sources. Produce could also be 'fresher' than retail outlets' produce. If this is the case, it could be argued that these strains might not survive that long on the surface of produce and would therefore be more common on recently harvested produce.

Cluster I consisted of the largest group of isolates and were all sourced from either farmers' markets or farms. In this cluster isolates 56, 55, 45, 44, 43, 42, 41, 39, 38, 37, 36, 32, 23, 22, 20 and 21 all had the same phenotypic characteristics but these isolates were sampled from a variety of produce including lettuce, parsley, cabbage and lettuce, and from a combination of farms and farmers' markets. The remaining isolates in this cluster could further be grouped into five smaller sub-groups.

The dendrogram in Fig. 9 was based on the S_{SM} coefficient method but only the 63 *E. coli* isolates were included together with the five ATCC reference stains. All isolates identified as species other than *E. coli* were excluded. The clusters are numbered in squares on the dendrogram from A to C.

The dendrogram (Fig. 9) only contains the *E. coli* isolates and showed three major clusters (A, B, C) and three ungrouped isolates which were considered outliers. The variance decomposition within the clusters was calculated as 56.09% (XLStat 2011.1.01) with the S_{SM} coefficient method according to Lockhart & Liston (1970).

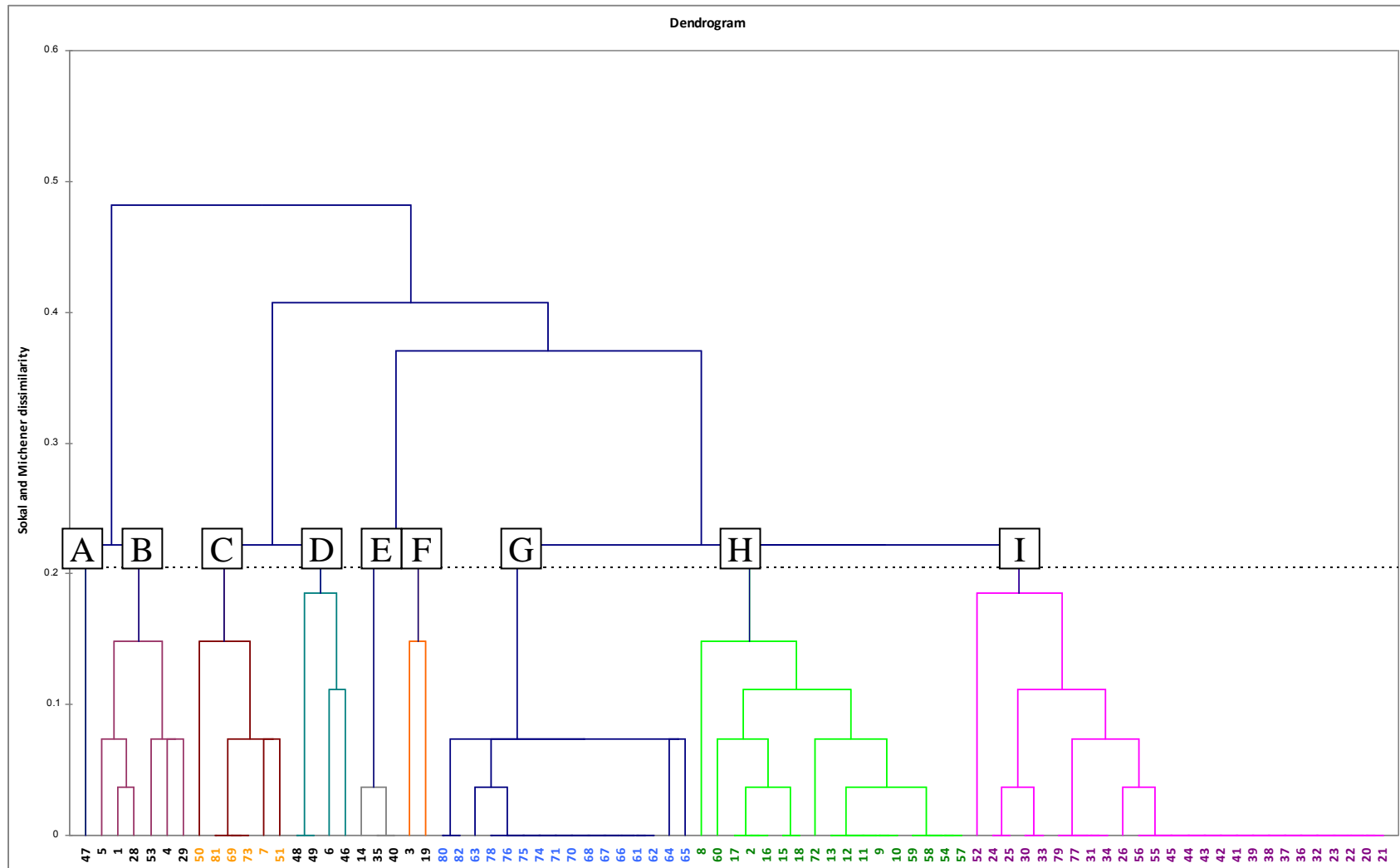


Figure 8 Simple matching coefficient dendrogram of all isolates based on the API 20E characters. Nine clusters were identified of which four (C, G, H, I) consisted of *E. coli* identified strains. The y-axis of the dendrogram represents the dissimilarity coefficient. The larger the factor and thus the distance between isolates and clusters, the greater the number of differences there are. On the x-axis all the isolate numbers are shown as well as their clusters grouping.

The largest cluster (A) with 43 of the 68 *E. coli* also contained the five ATCC reference strains. The isolates in this cluster were all sourced from produce from farms with the exception of six which were from a farmers' market (42, 41, 39, 38, 37 and 36). In cluster A, four of the five ATCC strains (R404, R158, R157 and R58) had the same phenotypic characteristics as isolates 79, 77, 31 and 34. Isolates 79 and 77 were from beans and isolates 31 and 34 were from cabbage. The fact that the ATCC strains and isolates from both the beans and cabbage sample have the same phenotypic characteristics indicates that the same type of *E. coli* can be present in more than one kind of location. This can make it difficult to control or back-track a specific *E. coli* type. Similarly, in cluster A, isolates 55, 56, 45, 44, 43, 42, 41, 39, 38, 37, 36, 32, 23, 22, 20 and 21 were all similar in phenotypic characteristics. The difference here was that these isolates were from a combination of products (parsley, lettuce, cabbage and spinach) sourced from both farms and farmers' markets. This is again a clear indication that *E. coli* with the same phenotypic characteristics can occur on a variety of produce types and also at different ecological sources.

In this study (Fig. 9) there were also incidences where *E. coli* with the same phenotypic characteristics (Cluster A) were found on the same produce type from the same source type. These were isolates 78, 76, 75, 74, 71, 70, 68, 67, 66, 61 and 62 that were all from beans sampled from different farms. It may be possible that there is a specific *E. coli* type that occurs only on beans or only on produce at PoH, but throughout this study the data shows that this was not the case.

All the isolates in cluster B (81, 69, 73, 72, 60, 13, 12, 11, 9, 10, 59, 58, 54 and 57) were sourced from farms and farmers' markets. From the data it was thus again concluded that there is not a specific phenotypic *E. coli* that could be associated with a specific produce type or specific produce source. Thus there remains a risk of the presence of pathogens, regardless of the source. The fact that one type of *E. coli* cannot be linked to only one produce type or from one source type, suggests that it will be more difficult to determine if there might be a pathogenic *E. coli* type that is limited on a specific produce type. Thus it poses a risk to the consumer when it is unknown on which produce specific pathogens are more prone to occur. The contamination source of the possible pathogen is also unpredictable and thus it adds to the risk of not being sure what can be present as the source of the possible pathogens is not specific.

In cluster C the nine isolates present were sourced from farmers' markets with the exception of two that were isolated from produce from farms (30 and 33). This could have been an indication that there is a certain type of *E. coli* present on fresh produce from farmers' markets but the presence of the two isolates sourced from farms counteracts it.

In Fig. 10, the S_J coefficient method, where only positive matches are considered, was used to construct the dendrogram which was again based on the API 20 characters of all the isolates (63) identified as *E. coli* with both the API and PCR systems. Three major clusters were found and they were numbered from A to C.

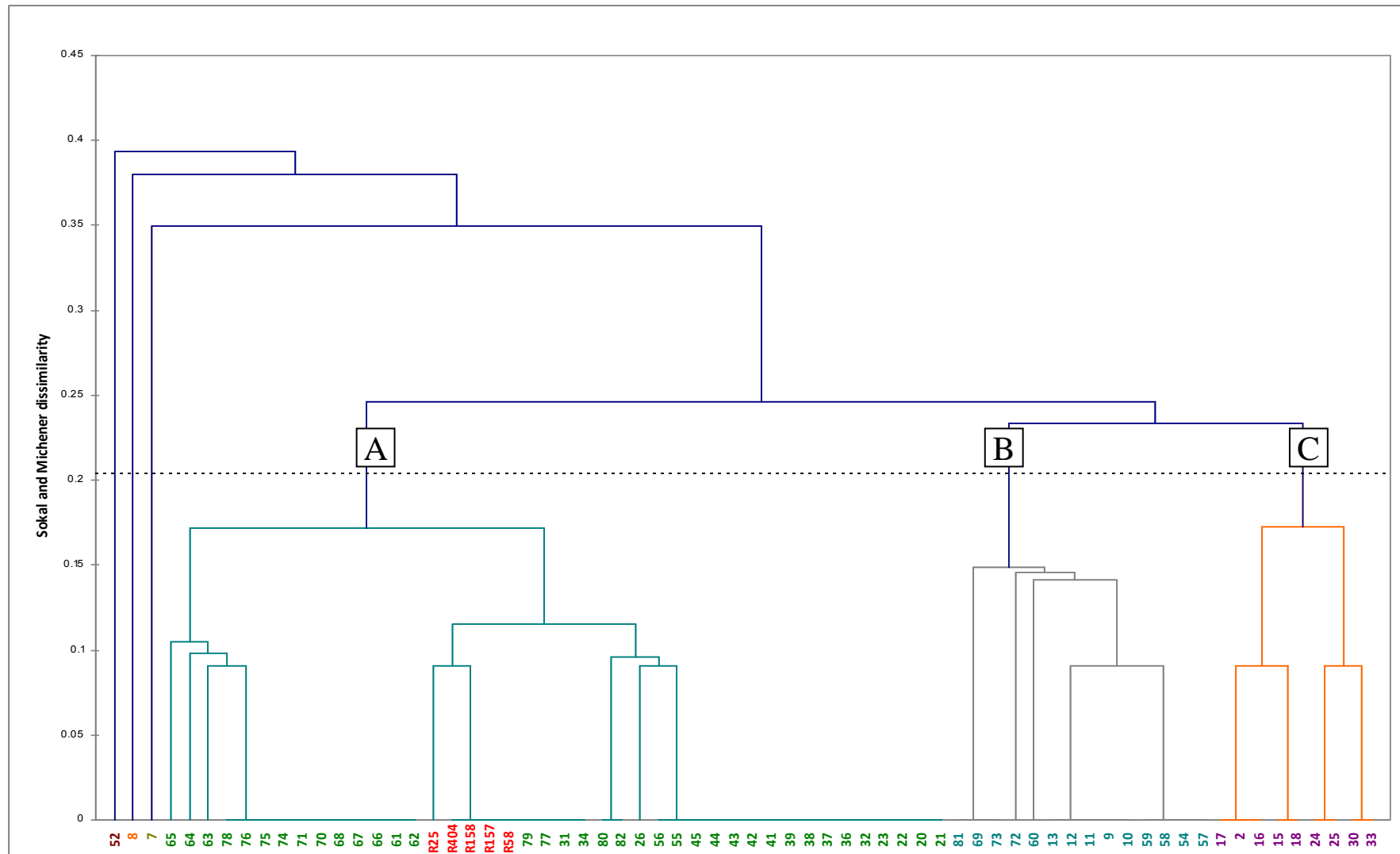


Figure 9 Simple matching coefficient dendrogram of isolates identified and confirmed with API 20E and *uidA* PCR as *E. coli*. The strains marked in red and starting with the letter R, were ATCC strains. The y-axis gives an indication of the dissimilarity coefficient. On the x-axis the *E. coli* isolate numbers are shown. The dotted horizontal line on the plot indicates the dissimilarity distance between the three clusters.

All the ATCC reference cultures (numbered in red) were grouped in a subsection of Cluster A. The other isolates in cluster A were from produce sourced from different farms. Isolate 52, which appears as an outlier of Cluster A, is the one exception that tested negatively for the characteristic ONPG (2-nitrophenyl- β D-galactopyranoside). Isolates 79, 77, 31, 34 and reference strains R404, R158, R157 and R58 were all phenotypically similar when applying the S_j coefficient (Fig. 10). Isolates 77 and 79 were strains from the Huisamen and Van Blommestein studies, which were originally sampled from beans (farms), while isolates 31 and 34 from this study were sampled from cabbage, also sourced from different farms. Again it was concluded that *E. coli* strains with the same characteristics are present at different locations. Isolates 78, 76, 75, 74, 71, 70, 68, 67, 66, 61 and 62 also all have the same phenotypic characteristics and were all sampled from beans sourced from different farms. In the case of isolate 63 only one characteristic (ODC - ornithine) was found to be negative, compared to isolates 78, 76, 75, 74, 71, 70, 68, 67, 66, 61 and 62 which were all positive for this characteristic.

The isolates in Cluster B were all isolated from either produce from farms, farmers' markets or from retail stores. Isolates 56, 55, 45, 44, 43, 42, 41, 39, 38, 37, 36, 32, 23, 22, 20 and 21 all had the same phenotypic characteristics and were sampled from a variety of produce (lettuce, parsley and cabbage) and from a combination of farm and farmers' markets. Isolates 7, 81, 69 and 73 are the only isolates in Cluster B that were negative for characteristic LDC (lysine) and thus they differ from the other isolates in Cluster B. The *E. coli* source variation of the isolates in Cluster B can possibly be explained in that there is not a specific *E. coli* strain that will only be present on produce from farms, retail outlets or farmers' markets.

Regarding the *E. coli* isolates having variation in characteristics it is supported as it also has been reported by Brennan *et al.* (2010) and Janezic *et al.* (2013). Brennan and associates found variation among *E. coli* isolates sourced from soil with characteristics ODC, SOR and SAC. Janezic *et al.* also found variation among *E. coli* isolates sourced from untreated surface water and differed in characteristics ONPG, LDC, ODC, MAN, RHA and SAC.

The isolates grouped in Cluster C were sourced from farms, farmers' markets and retail outlets and produce including parsley, beans and peas. The isolate combination again indicates that a certain *E. coli* cannot specifically be expected to be present on a certain produce type. There is a wide character variation in all three the Clusters in this dendrogram as the variance decomposition within clusters was statistically calculated (XLStat 2011.1.01) as 61.22% with the S_j coefficient method. The dissimilarity coefficient level where the three clusters are identified is 0.7. This is an indication that the variation of isolate characteristics is larger when only the positive results are taken into consideration.

PCA Analysis

A PCA (Principal Component Analysis) plot is a means of compressing a data table into a visible figure by arranging data in such a way that the scales measured showing the biggest variance are

visible on the plot (XLSTAT, Version 7.5.2, Addinsoft, New York, USA). By constructing the plot, all of the characteristics measured are taken into consideration and displayed in such a way to indicate the relationship to each other on these scales. A PCA plot will give a more visible interpretation of the relationship between the isolates in terms of their characteristics. The same API data for the positive identified *E. coli* were used for Figs. 9 and 10, to construct the PCA plot based on the S_j method. The PCA plot is illustrated in Fig. 11.

Correspondence analysis (CA) with TDA and ADH (Data not shown): In this case the PCA plot (not shown) showed that the TDA positive characteristic (Table 2) was only associated with isolate 8 and the ADH positive characteristic only associated with isolate 72. Note that this does not mean that isolates 8 or 72 only associate with the TDA or ADH characteristics. Thus, removing TDA and ADH characteristics from the analysis provided greater definition in the association between the rest of the characteristics and isolates (N. Ntushelo, Biometry Unit, ARC-Infruitec, Personal Communication, 2012).

Correspondence analysis (CA) without TDA and ADH (Fig. 11): The Clusters (A, B and C) showed in Fig. 10 can in most cases be directly linked to the Groups (A, B and C) in Fig. 11. Firstly, the same isolates were used to configure both Figs. 10 and 11 and secondly, the Clusters (Fig. 10) and Groups (Fig. 11) that formed consist mostly of the same combination of isolates. This plot (Fig. 11) shows the characteristics for which large variations were observed (SAC, ODC and VP) (Table 2) among the *E. coli* isolates and the main group of characteristics. The latter is a grouping of similar characters which include GLU, MAN, MEL, ARA, NO₂, McC, OF-O, OF-F (note that for these characters all strains gave similar results), and SOR, RHA, IND, LDC, ONPG, MOB (note that these show slight character combination variations – Table 2). Therefore we can refer to them as a grouping of similar characteristics clustered around the origin of the plot (double dotted circle) (N. Ntushelo, Biometry Unit, ARC-Infruitec, Personal Communication, 2012).

In the plot (Fig. 11) SAC is strongly associated with the isolates grouped closely to it (Group B) and additionally also includes isolates 82 and 80. Similarly, VP is strongly associated with isolates grouped closely to it (Group A) and again, includes isolates 82 and 80. ODC is also associated with isolates 82 and 80 and includes both groups of isolates (Groups A and B) that are associated with the SAC and VP characteristics. This also includes isolate 7 of Cluster B. Isolate 52 is clustered in Group B in Fig. 11 but is actually grouped within Cluster A when using the phenotypic characteristics as shown in Fig. 10.

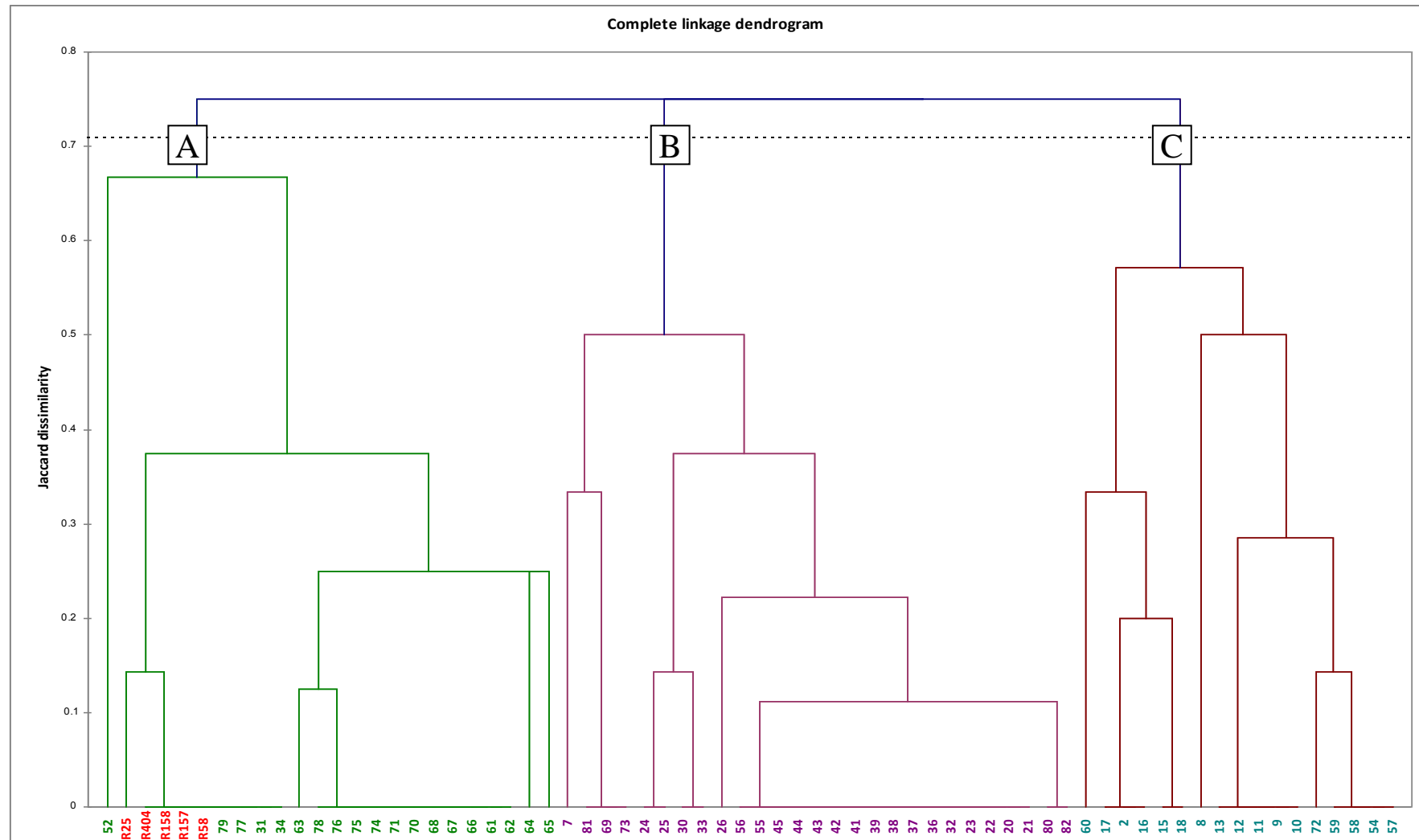


Figure 10 Jaccard coefficient dendrogram of *E. coli* isolates identified with both the API and *uidA* PCR system. The y-axis gives an indication of the dissimilarity coefficient level. On the x-axis all the isolates numbers are listed. The dotted horizontal line on the plot indicates the dissimilarity distance between the three clusters which in this case does not indicate a close relation between the clusters. The strains marked in red and starting with the letter R, are the ATCC strains.

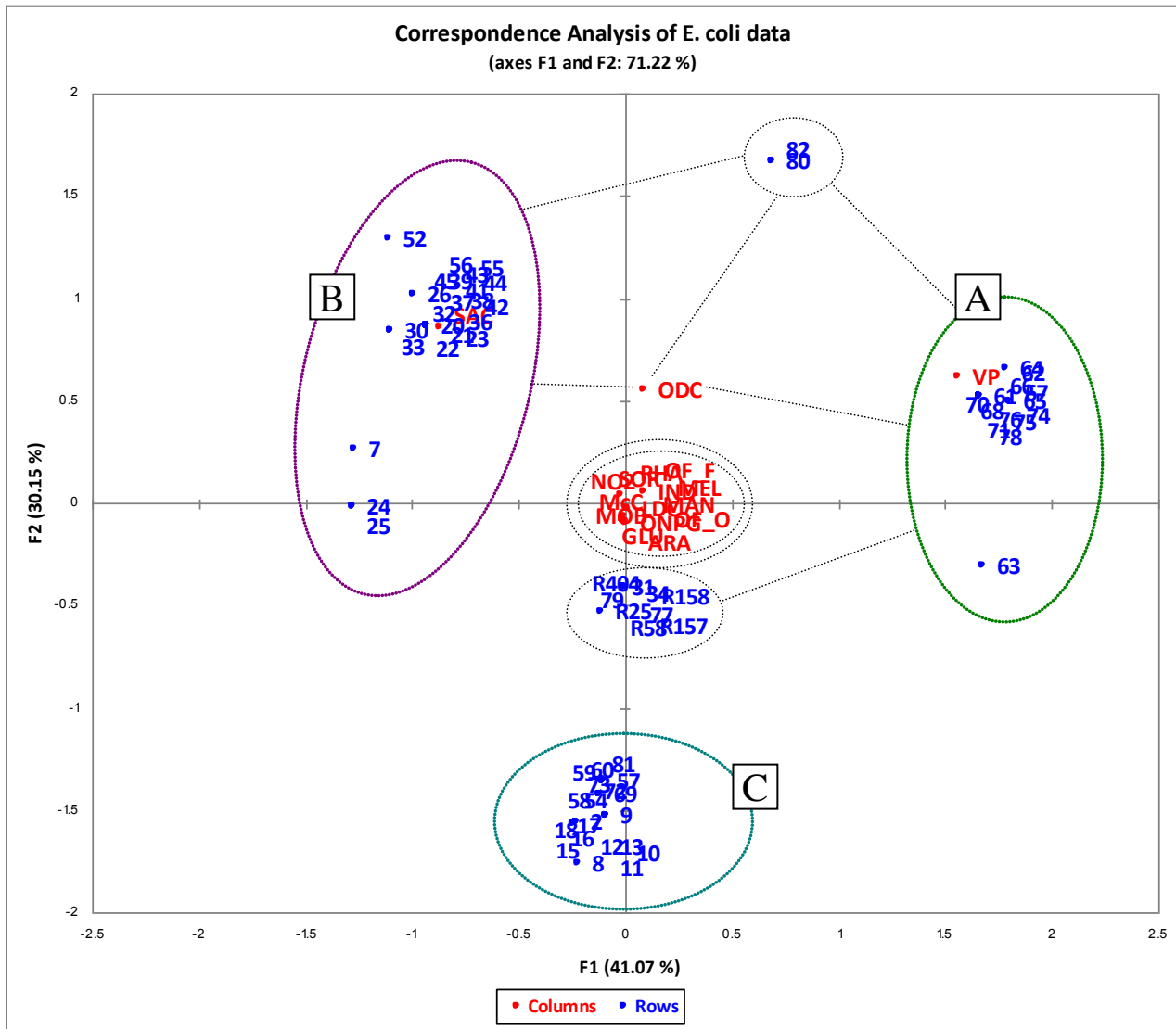


Figure 11 PCA plot of all the positively identified *E. coli* identified isolates based on the S_J dendrogram. The API 20E data was used to construct the plot. The plot was constructed without the TDA and ADH characters. Three major groups can be identified and are marked from A to C. The dotted lines indicate isolates that form subgroups of Groups A to C.

Isolates 24 and 25 (of Group B) only associate with SAC and most of the characteristics clustered around the origin (double dotted circle). Isolate 63 associates with VP (Group A) and most of the characteristics that are clustered around the origin (double dotted circle).

Group C and the group of ATCC reference strains and a few isolates that grouped above Group C (Fig. 11) only associates with the characteristics grouped in the origin (double dotted circle) and not with ODC, SAC or VP.

Table 2 API 20E characters and character descriptions with the percentage positive reactions for each character in each major cluster

Character	Description	Cluster A	Cluster B	Cluster C
ONPG	β -galactosidase (Ortho NitroPhenyl- β D-Galactopyranosidase)	96	100	100
ADH	Arginine DiHydrolase	0	0	6
LDC	Lysine DeCarboxylase	100	85	100
ODC	Ornithine DeCarboxylase	96	78	0
CIT	Citrate utilization	0	0	0
H ₂ S	H ₂ S production	0	0	0
URE	Urease	0	0	0
TDA	Tryptophane deaminase	0	0	6
IND	Indole production	96	85	65
VP	Acetoin production (Voges Proskauer)	58	7	0
GEL	Gelatinase	0	0	0
GLU	Fermentation/oxidation glucose	100	100	100
MAN	Fermentation/oxidation mannitol	100	100	100
INO	Fermentation/oxidation inositol	0	0	0
SOR	Fermentation/oxidation sorbitol	100	96	100
RHA	Fermentation/oxidation rhamnose	92	100	65
SAC	Fermentation/oxidation saccharose	4	89	0
MEL	Fermentation/oxidation melibiose	100	100	100
AMY	Fermentation/oxidation amygdalin	0	0	0
ARA	Fermentation/oxidation arabinose	100	100	100
OX	Cytochrome-oxidase	0	0	0
NO ₂	NO ₂ production	100	100	100
N ₂	Reduction to N ₂ gas	0	0	0
MOB	Mobility	92	96	82
McC	Growth	100	100	100
OF-O	Oxidation	100	100	100
OF-F	Fermentation	100	100	100

All the isolates in Group A in Fig. 11 are positive for VP. These isolates in Group A in Fig. 11 are all positive for most of the characteristics marked in red in the middle (double dotted circle) of the PCA plot.

All the ATCC reference strains, R404, R25, R58, R157 and R158 together with isolates 77, 79, 31 and 34 are situated near the centre of the plot but can also be sub-grouped to Group A. This is also shown in Fig. 10 as part of Cluster A.

From the data in Fig. 11 it is clear that there are three distinctive major Groups of *E. coli* types (A, B and C) present and they are confirmed as members of the Clusters of the dendrogram in Fig.10. It is also clear that the PCA plot is of value in displaying and confirming the phenotypic relationships between the major clusters found in this study. There is also an indication of minor phenotypic variation when evaluating the PCA placing of some of the isolates. It is of interest that the three major Groups cannot be assigned as a group to a specific source type or produce type and thus this makes it very difficult to assume that a cluster of *E. coli* strains pose a direct health risk to the consumer.

CONCLUSIONS

In this study the washing of the surface of the produce was found to be the most effective TC and *E. coli* enumeration method to determine the microbial level on the surface of the fresh produce. However, this method of washing the fresh produce samples ensures a representation of the surface bacteria only and not the total of the whole produce. As the study is part of a larger project investigating polluted river water used for irrigation purposes, the presence of *E. coli* was only investigated on the surface of the produce.

The overall TC levels found on the fresh produce sampled was high as the majority of the samples tested exceed the recommended guidelines of the DoH (DoH, 2011). According to DWAF, such high TC levels are considered as a significant and increasing risk for infectious disease transmission (DWAF, 1996). Thus the high TC levels found on the majority of the fresh produce samples can be considered a definite risk indicator for consumer health.

Overall the data from the study showed the presence of *E. coli* on the produce was not as widespread in terms of produce sources as expected but they were certainly present. According the DoH recommended guidelines *E. coli* must be absent on vegetables to be consumed raw (DoH, 2011). Thus the presence of *E. coli* should be seen as unacceptable as it indicates a risk to the consumer.

In terms of the presence of *E. coli* on produce, *E. coli* was detected on samples from all the source types including the farms, farmers' markets and retail outlets but were the most prevalent on produce from farmers' markets. *Escherichia coli* was present on 12 of the 151 produce samples sampled during this study. This is 8% of the produce samples that indicated the presence of *E. coli*.

Three clear Clusters were formed when the *E. coli* data was statistically analysed and presented in dendrograms. Clear groups of *E. coli* were identified indicating that there are *E. coli* strains with similar phenotypic characteristics. These groups could however not be assigned to only one produce type or one source type.

Ultimately it was concluded that a specific *E. coli* cannot be assigned to a certain produce type. *Escherichia coli* were found present on the fresh produce and thus it is of concern to the consumer. This indicates that it cannot be predicted that a certain type of pathogenic *E. coli* is likely to be present on a certain produce type or on produce from a certain source including farms, farmers' markets or retail outlets. Thus the unpredictable presence of *E. coli* can be perceived as a risk to the consumer but also as a positive observation as no clear contamination source could be identified, decreasing the chance of a localised disease outbreak.

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Addendum A

The API 20E profile, percentage identification for each isolate and their source, produce type and *uidA* PCR result

Isolate #	Source	Produce	API 20 E Identification	Profile	API 20E %	<i>uidA</i> confirmation
1	Retail 3	Lettuce	<i>Raoultella terrigena</i>	Doubtful profile	71.4	-
2	Retail 3	Beans	<i>Escherichia coli 1</i>	Good identification	96.8	<i>E. coli</i>
3	Retail 3	Lettuce	<i>Enterobacter cancerogenus</i>	Good identification	98.7	-
4	Retail 3	Beans	no ID	Low discrimination	-	-
5	Retail 3	Beans	<i>Klebsiella pneumoniae ssp pneumoniae</i>	Doubtful profile	97.4	-
6	Retail 3	Lettuce	<i>Klebsiella pneumoniae ssp ozaenae</i>	Good identification	91.3	-
7	Retail 3	Beans	no ID	Low discrimination	-	<i>E. coli</i> *
8	Market 1	Peas	<i>Escherichia coli 1</i>	Doubtful profile	98.3	<i>E. coli</i>
9	Market 1	Peas	<i>Escherichia coli 1</i>	Excellent identification	99.9	<i>E. coli</i>
10	Market 1	Peas	<i>Escherichia coli 1</i>	Excellent identification	99.9	<i>E. coli</i>
11	Market 1	Peas	<i>Escherichia coli 1</i>	Excellent identification	99.9	<i>E. coli</i>
12	Market 1	Peas	<i>Escherichia coli 1</i>	Excellent identification	99.9	<i>E. coli</i>
13	Market 1	Peas	<i>Escherichia coli 1</i>	Excellent identification	99.9	<i>E. coli</i>
14	Market 3	Peas	<i>Enterobacter cloacae</i>	Doubtful profile	95.0	-
15	Market 3	Peas	no ID	Low discrimination	-	<i>E. coli</i> *
16	Market 3	Peas	<i>Escherichia coli 1</i>	Good identification	96.8	<i>E. coli</i>
17	Market 3	Peas	<i>Escherichia coli 1</i>	Good identification	96.8	<i>E. coli</i>
18	Market 3	Peas	no ID	Low discrimination	-	<i>E. coli</i> *
19	Retail 1	Beans	<i>Kluyvera spp</i>	Good identification	98.2	-
20	Farm 2	Cabbage	<i>Escherichia coli 1</i>	Very good identification	99.5	<i>E. coli</i>
21	Farm 2	Cabbage	<i>Escherichia coli 1</i>	Very good identification	99.5	<i>E. coli</i>
22	Farm 2	Cabbage	<i>Escherichia coli 1</i>	Very good identification	99.5	<i>E. coli</i>

Isolate #	Source	Produce	API 20 E Identification	Profile	API 20E %	<i>uidA</i> confirmation
23	Farm 2	Cabbage	<i>Escherichia coli</i> 1	Very good identification	99.5	<i>E. coli</i>
24	Market 3	Beans	<i>Escherichia coli</i> 1	Very good identification	99.1	<i>E. coli</i>
25	Market 3	Beans	<i>Escherichia coli</i> 1	Very good identification	99.1	<i>E. coli</i>
26	Market 6	Lettuce	<i>Escherichia coli</i> 1	Very good identification	99.4	<i>E. coli</i>
28	Market 6	Lettuce	<i>Klebsiella pneumoniae</i> spp <i>pneumoniae</i>	Doubtful profile	97.7	-
29	Market 6	Lettuce	<i>Klebsiella pneumoniae</i> spp <i>pneumoniae</i>	Good identification	97.7	-
30	Farm 3	Cabbage	<i>Escherichia coli</i> 1	Good identification	98.5	<i>E. coli</i>
31	Farm 3	Cabbage	<i>Escherichia coli</i> 1	Excellent identification	99.9	<i>E. coli</i>
32	Farm 3	Cabbage	<i>Escherichia coli</i> 1	Very good identification	99.5	<i>E. coli</i>
33	Farm 3	Cabbage	<i>Escherichia coli</i> 1	Good identification	98.5	<i>E. coli</i>
34	Farm 3	Cabbage	<i>Escherichia coli</i> 1	Excellent identification	99.9	<i>E. coli</i>
35	Farm 3	Cabbage	<i>Enterobacter cloacae</i>	Good identification	95.0	-
36	Market 2	Spinach	<i>Escherichia coli</i> 1	Very good identification	99.5	<i>E. coli</i>
37	Market 2	Spinach	<i>Escherichia coli</i> 1	Very good identification	99.5	<i>E. coli</i>
38	Market 2	Spinach	<i>Escherichia coli</i> 1	Very good identification	99.5	<i>E. coli</i>
39	Market 2	Spinach	<i>Escherichia coli</i> 1	Very good identification	99.5	<i>E. coli</i>
40	Market 2	Spinach	<i>Enterobacter cloacae</i>	Good identification	95.0	-
41	Market 2	Spinach	<i>Escherichia coli</i> 1	Very good identification	99.5	<i>E. coli</i>
42	Market 2	Spinach	<i>Escherichia coli</i> 1	Very good identification	99.5	<i>E. coli</i>
43	Market 6	Lettuce	<i>Escherichia coli</i> 1	Very good identification	99.5	<i>E. coli</i>
44	Market 6	Lettuce	<i>Escherichia coli</i> 1	Very good identification	99.5	<i>E. coli</i>
45	Market 6	Lettuce	<i>Escherichia coli</i> 1	Very good identification	99.5	<i>E. coli</i>
46	Farm 5	Beans	<i>Pantoea</i> spp 3	Good identification	95.7	-
47	Farm 5	Beans	<i>Serratia plymuthica</i>	Good identification to the genus	64.4	-
48	Farm 5	Beans	<i>Pantoea</i> spp 4	Doubtful profile	97.3	-

Isolate #	Source	Produce	API 20 E Identification	Profile	API 20E %	<i>uidA</i> confirmation
49	Farm 5	Beans	<i>Pantoea spp 4</i>	Doubtful profile	97.3	-
50	Farm 5	Beans	no ID	Low discrimination	-	-
51	Farm 5	Beans	no ID	Low discrimination	-	-
52	Farm 5	Parsley	<i>Escherichia coli 1</i>	Good identification to the genus	95.7	<i>E. coli</i>
53	Farm 5	Parsley	<i>Klebsiella oxytoca</i>	Good identification	97.4	-
54	Farm 5	Parsley	<i>Escherichia coli 1</i>	Excellent identification	99.9	<i>E. coli</i>
55	Farm 5	Parsley	<i>Escherichia coli 1</i>	Very good identification	99.5	<i>E. coli</i>
56	Farm 5	Parsley	<i>Escherichia coli 1</i>	Very good identification	99.5	<i>E. coli</i>
57	Farm 5	Parsley	<i>Escherichia coli 1</i>	Excellent identification	99.9	<i>E. coli</i>
58	Farm 5	Parsley	<i>Escherichia coli 1</i>	Excellent identification	99.9	<i>E. coli</i>
59	Farm 5	Parsley	<i>Escherichia coli 1</i>	Excellent identification	99.9	<i>E. coli</i>
60	Farm 5	Parsley	<i>Escherichia coli 1</i>	Good identification	97.1	<i>E. coli</i>
61	Farm	Beans	<i>Escherichia coli 1</i>	Doubtful profile	99.9	<i>E. coli</i>
62	Farm	Beans	<i>Escherichia coli 1</i>	Doubtful profile	99.9	<i>E. coli</i>
63	Farm	Beans	<i>Escherichia coli 1</i>	Doubtful profile	99.6	<i>E. coli</i>
64	Farm	Beans	<i>Escherichia coli 1</i>	Doubtful profile	99.5	<i>E. coli</i>
65	Farm	Beans	<i>Escherichia coli 1</i>	Doubtful profile	99.9	<i>E. coli</i>
66	Farm	Beans	<i>Escherichia coli 1</i>	Doubtful profile	99.9	<i>E. coli</i>
67	Farm	Beans	<i>Escherichia coli 1</i>	Doubtful profile	99.9	<i>E. coli</i>
68	Farm	Beans	<i>Escherichia coli 1</i>	Doubtful profile	99.9	<i>E. coli</i>
69	Farm	Beans	<i>Escherichia coli 1</i>	Good identification	97.4	<i>E. coli</i>
70	Farm	Beans	<i>Escherichia coli 1</i>	Doubtful profile	99.9	<i>E. coli</i>
71	Farm	Beans	<i>Escherichia coli 1</i>	Doubtful profile	99.9	<i>E. coli</i>
72	Farm	Beans	<i>Escherichia coli 1</i>	Very good identification	99.8	<i>E. coli</i>
73	Farm	Beans	<i>Escherichia coli 1</i>	Good identification	97.4	<i>E. coli</i>

Isolate #	Source	Produce	API 20 E Identification	Profile	API 20E %	<i>uidA</i> confirmation
74	Farm	Beans	<i>Escherichia coli</i> 1	Doubtful profile	99.9	<i>E. coli</i>
75	Farm	Beans	<i>Escherichia coli</i> 1	Doubtful profile	99.9	<i>E. coli</i>
76	Farm	Beans	<i>Escherichia coli</i> 1	Doubtful profile	99.9	<i>E. coli</i>
77	Farm	Beans	<i>Escherichia coli</i> 1	Excellent identification	99.9	<i>E. coli</i>
78	Farm	Beans	<i>Escherichia coli</i> 1	Doubtful profile	99.9	<i>E. coli</i>
79	Farm	Beans	<i>Escherichia coli</i> 1	Excellent identification	99.9	<i>E. coli</i>
80	Farm	Beans	<i>Escherichia coli</i> 1	Doubtful profile	99.2	<i>E. coli</i>
81	Farm	Beans	<i>Escherichia coli</i> 1	Good identification	97.4	<i>E. coli</i>
82	Farm	Beans	<i>Escherichia coli</i> 1	Doubtful profile	99.2	<i>E. coli</i>

*Isolate not identified as *E. coli* with API 20E but confirmed to be *E. coli* with *uidA* PCR

- = negative for *uidA* presence

CHAPTER 4

DETERMINATION OF *ESCHERICHIA COLI* PHYLOGENETIC GROUPS, INTESTINAL AND EXTRAIESTINAL PATHOGENIC *E. COLI* PRESENT ON FRESH PRODUCE

SUMMARY

Sixty-three *E. coli* isolates were subjected to triplex and multiplex PCR methods to identify their phylogenetic groups and the presence of intestinal pathogenic *E. coli* and extraintestinal pathogenic *E. coli* strains. Phylogenetic grouping revealed that the isolates belonged to five of the seven possible phylogenetic sub-groups. A total of 14 isolates belonged to genogroup A₀, 11 to A₁, 20 to B₁, 7 to B₂₃ and 11 to D₂. Multiplex PCR testing for INPEC revealed that none of the *E. coli* isolates were carriers of the INPEC gene sequences *ial*, *stx 1*, *stx 2*, *eagg*, *eaeA*, *LT* and *ST*. The *E. coli* isolates in this study were also submitted to multiplex PCR testing for the presence of the statistically most abundant ExPEC gene sequences: *papA*, *papC*, *sfa/foc*, *iutA*, *kpsMT II* and *afa/dra*. None of the isolates in this study were classified as ExPEC (which required the presence of two or more genes). Three of the isolates did however test positive for the presence of the *kpsMT II* gene. The latter is a group 2 capsular polysaccharide unit giving *E. coli* the ability to synthesise a capsule around its surface which is known to add to the bacterium's virulence as it enhances the bacterium's survival. Phagocytosis will be prevented by this capsule and thus the bacteria cannot be easily inactivated in the human body. To conclude, the presence of *E. coli*, commensal or pathogenic, is unacceptable. Thus the presence of a pathogenic gene, in this case an ExPEC gene, poses an even bigger risk to the consumer regarding the safety of fresh produce.

INTRODUCTION

Fresh produce is commonly accepted as a healthy food product. Foodborne outbreaks linked to fresh produce are increasing and *E. coli* has been shown to be a major cause of these outbreaks (Lynch *et al.*, 2009). *Escherichia coli* is mostly harmless to the consumer as it is a natural inhabitant of the human intestinal gut in its commensal form. Several foodborne pathogenic *E. coli* strains have, however, been identified and include strains O26, O157:H7, O104:H4 and O145 (CDC, 2010; CDC, 2011; CDC, 2012a; CDC, 2012b; CDC, 2012c). The best known *E. coli* pathogen is Enterohemorrhagic *E. coli* O157:H7 which is frequently tested for as a safety measure for food products (Gilbert *et al.*, 2000; HPA, 2009). In 2011, a major outbreak occurred after widespread consumption of sprouts in Germany. This outbreak was caused by *E. coli* O104:H4, which was classified as both an Enteroaggregative *E. coli* and Enterohemorrhagic *E. coli* strain (Warriner, 2011).

It has been reported that little is known about the variation of bacterial communities on different produce types except that they do differ between produce types (Leff & Fierer, 2013). *Escherichia coli* have been reported on lettuce, parsley and various other vegetables throughout Africa (Okafu *et al.*, 2003; Chigor *et al.*, 2010). Similarly the Shiga toxin-producing *E. coli* has been

found on sprouts, spinach, hazelnuts and lettuce (CDC, 2010; CDC, 2011; Warriner, 2011; CDC, 2012a; CDC, 2012b; CDC, 2012c). The Enterohemorrhagic *E. coli* has been reported to be present in water and on irrigated vegetables (Okafo *et al.*, 2003). It has also been shown that foodborne disease outbreaks linked to vegetables are mostly due to faecal contamination of irrigation water (Okafo *et al.*, 2003).

Escherichia coli can be placed in phylogenetic and pathogenic groups. The four phylogenetic groups (A, B1, B2 and D) can further be divided into seven sub-groups: A₀, A₁, B1, B2₂, B2₃, D₁ and D₂ (Lecointre *et al.*, 1998; Gordon *et al.*, 2008; Carlos *et al.*, 2010). The four different phylogenetic groups can be distinguished in terms of their environmental niches and their tendency to carry virulence genes (Gordon *et al.*, 2008; Carlos *et al.*, 2010). The pathogenic groups can be identified by the presence of certain virulence genes. The commensal *E. coli*, intestinal pathogenic *E. coli* (INPEC) and extraintestinal pathogenic *E. coli* (ExPEC) can also be grouped roughly into the phylogenetic groups (Pupo *et al.*, 1997; Johnson *et al.*, 2001). The commensal *E. coli* is most often placed in the A and B1 groups (Johnson *et al.*, 2001). Intestinal pathogenic *E. coli* strains are usually placed in the phylogenetic groups A, B1 and D (Pupo *et al.*, 1997) while the ExPEC strains usually cluster in phylogenetic group B2 and on occasion in group D (Johnson *et al.*, 2001).

Intestinal pathogenic *E. coli* consist of Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC), Enteraggregative *E. coli* (EAEC) and Diffusely Adherent *E. coli* (DAEC) (Donneberg & Kaper, 1992; O'Brien & Holmes, 1996; Scaletsky *et al.*, 2002; Kaper *et al.*, 2004). Extraintestinal pathogenic *E. coli* consist of Uropathogenic (UPEC), Sepsis-associated (SEPEC) and Neonatal meningitis associated (NEMEC) strains (Johnson *et al.*, 2001; Johnson & Russo, 2002; Russo & Johnson, 2009). If irrigation water is contaminated and used for fresh produce irrigation, the presence of pathogenic *E. coli* will most likely be transferred to the fresh produce. Since there are several South African reports (Lötter, 2010; Gemmell & Schmidt, 2012) of irrigation water being faecally contaminated, it is important to know which phylogenetic and pathogenic *E. coli* are present on produce so as to determine if there is a risk to the consumer and then to reduce or eliminate the risk if present.

The aim of this research is to determine the types of *E. coli* present on fresh produce in terms of the phenotypic and phylogenetic properties as well as potential for intestinal and extraintestinal pathogenicity. Each *E. coli* isolate positively identified with both the API 20E and *uidA* PCR analysis in the previous chapter will be evaluated using triplex PCR to determine the phylogenetic groupings. Multiplex PCR analysis will also be applied to determine if pathogenic genes (for INPEC and ExPEC) are present.

MATERIALS AND METHODS

***Escherichia coli* strains**

In the previous chapter of this thesis 63 *E. coli* were isolated from fresh produce samples sourced from farms, farmers' markets and retail outlets. Each isolate was identified using the API 20E system and then the strains' identity as *E. coli* was confirmed with PCR (presence of the *uidA* gene). In this chapter each confirmed *E. coli* isolate was tested further to determine which phylogenetic group they belong to and to determine if they are intestinal pathogenic or extraintestinal pathogenic *E. coli*.

DNA template preparation

Each isolate was cultivated on Tryptone Soya Agar (TSA) (Oxoid) at 37°C for 24 h. From the TSA plates, a colony was transferred to a 1.5 mL microcentrifuge tube (Quality Scientific Plastics®) already containing 100 µL sterile nuclease-free water. The cell suspension was boiled for 13 min, cooled on ice and centrifuged at 14 000 x g for 15 min. The supernatant was transferred to a 0.6 µL sterile centrifuge tube and then stored at -18°C (Altahi & Hassen, 2009).

Polymerase Chain Reaction (PCR) analysis

Genotype PCR

All the *E. coli* isolates were tested using the triplex PCR method of Clermont *et al.* (2000) to determine *E. coli* genotypes. Three fragments were amplified; *chuA*, *yjaA* and *TSPE4.C2*. For this study each reaction tube contained 6.25 µL of 1 x KAPA2G Fast Multiplex Mix, 0.2 µM of each primer (Table 2) and 0.25 µL of template DNA. Nuclease-free water was added to have a total reaction volume of 12.5 µL.

PCR reactions were performed using a G-storm thermal cycler (Vacutec). The operational conditions were as follows: initial denaturation for 3 min at 95°C; 30 cycles of denaturation for 30 s at 95°C, primer annealing for 30 s at 60°C, extension for 30 s at 72°C; followed by a final extension for 5 min at 72°C. A positive control (with template DNA from *E. coli* strain ATCC 25922), and a negative control (with nuclease-free water instead of template DNA), were included with all PCR reactions.

Gel electrophoresis was done to view the PCR products with a 2% agarose (SeeKem) gel containing 1 µg.mL⁻¹ ethidium bromide (Sigma). The gel electrophoresis was carried out with a Baygene-BG-Power300 power supply (Vacutec, South Africa) at 210 V for 20 min. The bands were visualised using a UV transilluminator (Vacutec, South Africa).

In Table 1 all the phylogenetic groups of *E. coli* that can be determined with the method of Clermont *et al.* (2000), are represented.

Table 1 Phylogenetic group classification based on PCR detection method of Clermont *et al.* (2000)

Gene sequence	Phylogenetic group						
	A ₀	A ₁	B1	B2 ₂	B2 ₃	D ₁	D ₂
<i>chuA</i>	-	-	-	+	+	+	+
<i>yjaA</i>	-	+	-	+	+	-	-
TSPE ₄ C ₂	-	-	+	-	+	-	+

(Clermont *et al.*, 2000; Carlos *et al.*, 2010)

INPEC Multiplex PCR

All the *E. coli* isolates were tested for the presence of INPEC gene sequences according to a method modified from Omar & Barnard (2010). With this PCR, eight genes were amplified; *mdh*, *eagg*, *stx1*, *stx2*, *ial*, *eaeA*, *LT* and *ST*. Each reaction tube contained 6.25 µL of 1 x KAPA2G Fast Multiplex Mix, 0.2 µM of each primer (Table 2) and 0.25 µL of template DNA. Nuclease-free water was added up to a total volume of 12.5 µL.

Positive and negative controls were included in all PCR's. The negative control had nuclease-free water instead of template DNA. The positive control contained equal volumes of DNA of EAEC, EHEC, EIEC, EPEC and ETEC standard cultures, kindly provided by Dr. T.G. Barnard, Faculty of Health Sciences, University of Johannesburg.

All PCR reactions were performed in a G-storm thermal cycler (Vacutec, South Africa). The reaction conditions were as follows: initial denaturation for 3 min at 95°C; 30 cycles of denaturation for 15 s at 95°C, primer annealing for 30 s at 55°C, extension for 30 s at 68°C; followed by a final extension for 5 min at 72°C.

Gel electrophoresis was done to visualise the PCR products using a 1.25% agarose (SeeKem) gel containing 1 µg.mL⁻¹ ethidium bromide (Sigma). The gel electrophoresis was carried out with a Baygene-BG-Power300 power supply (Vacutec) for 90 min. at 120 V and the bands visualised with a UV transilluminator (Vacutec, South Africa).

ExPEC Multiplex PCR

All the *E. coli* isolates were tested for the presence of ExPEC using the method of Xia *et al.*, (2011). Six sequences were amplified; *papA*, *papC*, *sfa/foc*, *iutA*, *kpsMT II* and *afa/dra*. These six genes have been statistically proven to be the most common in ExPEC strains and from these six genes, two or more should be present for an isolate to be classified as an ExPEC (Xia *et al.*, 2011). The reaction tube contained 6.25 µL of 1x KAPA2G Fast Multiplex Mix, 0.25 µL of primer mix which contain 0.2 µM of each primer (Table 2) and 0.25 µL of template DNA. Nuclease-free water is added to add up the total volume to 12.5 µL.

Table 2 Primer sequences for each gene amplification in the PCR methods

GENE/PATHOTYPE	PRIMER*	SIZE (BP)	PRIMER SEQUENCE (5'-3')	REFERENCE
Genogroups method (Clermont <i>et al.</i>, 2000)				
<i>yjaA</i>	YJAA.1 (f)	211	TGAAGTGTCCAGGAGACGCTG	Clermont <i>et al.</i> , 2000
	YJAA.2 (r)		ATGGAGAATGCGTTCCCTCAAC	
<i>chuA</i>	CHUA.1 (f)	279	GACGAACCAACGGTCAGGAT	Clermont <i>et al.</i> , 2000
	CHUA.2 (r)		TGCCGCCAGTACCAAAGACA	
TSPE4.C2	TSPE4.C2.1 (f)	152	GAGTAATGTCGGGGCATTCA	Clermont <i>et al.</i> , 2000
	TSPE4.C2.2 (r)		CGCGCCAACAAAGTATTACG	
INPEC method (modified from Omar & Barnard, 2010)				
<i>mdh</i> (commensal)	MDH01	300	GGTATGGATCGTTCCGACCT	Tarr <i>et al.</i> , 2002
	MDH02		GGCAGAATGGTAACACCAGAGT	
<i>eagg</i> (EAEC)	EAGG(f)	194	AGACTCTGGCGAAAGACTGTATC	Pass <i>et al.</i> , 2000
	EAGG(r)		ATGGCTGTCTGTAATAGATGAGAAC	
<i>stx 1, stx 2</i> (EHEC)	STX1(f)	614	ACACTGGATGATCTCAGTGG	Moses <i>et al.</i> , 2006
	STX1(r)		CTGAATCCCCCTCCATTATG	
	STX2(f)	779	CCATGACAACGGACAGCAGTT	Moses <i>et al.</i> , 2006
	STX2(r)		CCTGTCAACTGAGCACTTTG	
<i>ial</i> (EIEC)	L-IAL(f)	650	GGTATGATGATGATGAGTCCA	Lopez-Suacedo <i>et al.</i> , 2003
	IAL(r)		GGAGGCCAACAATTATTTCC	
<i>eeA</i> (EPEC/EHEC)	L-EAEA(f)	384	GACCCGGCACAAGCATAAGC	Lopez-Suacedo <i>et al.</i> , 2003
	L-EAEA(r)		CCACCTGCAGCAACAAGAGG	
<i>LT, ST</i> (ETEC)	LT(f)	450	GGCGACAGATTATACCGTGC	Lopez-Suacedo <i>et al.</i> , 2003
	LT(r)		CGGTCTCTATATCCCTGTT	
	ST(f)	160	TTTCCCCTCTTTTAGTCAGTCAACT	Omar & Barnard, 2010
	ST(r)		GGCAGGATTACAACAAAGTTCACA	
ExPEC method (Xia <i>et al.</i>, 2011)				
<i>papA</i>	PAPA(f)	717	ATGGCAGTGGTGTCTTTTGGTG	Johnson <i>et al.</i> , 2003
	PAPA(r)		CGTCCCACCATACGTGCTCTTC	
<i>papC</i>	PAPC(f)	205	GTGGCAGTATGAGTAATGACCGTTA	Johnson <i>et al.</i> , 2003
	PAPC(r)		ATATCCTTTCTGCAGGGATGCAATA	
<i>sfa/foc</i>	SFA1 (f)	410	CTCCGGAGAAGTGGGTGCATCTTAC	Johnson <i>et al.</i> , 2003
	SFA2 (r)		CGGAGGAGTAATTACAAACCTGGCA	
<i>iutA</i>	IUTA(f)	302	GGCTGGACATCATGGGAAGTGG	Johnson <i>et al.</i> , 2003
	IUTA(r)		CGTCGGGAACGGGTAGAATCG	
<i>kpsMT II</i>	KPSII(f)	272	GCGCATTTGCTGATACTGTTG	Johnson <i>et al.</i> , 2003
	KPSII(r)		CATCCAGACGATAAGCATGAGCA	
<i>afa/dra</i>	AFA(f)	592	GGCAGAGGGCCGGCAACAGGC	Johnson <i>et al.</i> , 2003
	AFA(r)		CCCCTAACGCGCCAGCATCTC	

*f – forward primer, r – reverse primer

All the PCR reactions were performed in a G-storm thermal cycler (Vacutec). The reaction conditions were as follows: initial denaturation for 3 min at 95°C, 30 cycles of denaturation for 15 s at 95°C, primer annealing for 30 s at 61°C, extension for 30 s at 68°C, followed by a final extension for 5 min at 72°C and cooling for 2 min at 4°C.

Gel electrophoresis was done to visualise the PCR products on a 1.25% agarose (SeeKem) gel containing 1 µg.mL⁻¹ ethidium bromide (Sigma). The gel electrophoresis was carried out with a Baygene-BG-Power300 power supply (Vacutec) for 90 min. at 120 V and the bands visualised with a UV transilluminator (Vacutec, South Africa).

The negative control had nuclease-free water instead of template DNA. The positive control consists of equal volumes of ATCC 25922 and clinical isolate JUL 1211. These two strains were decided on after screening *E. coli* isolates for the applicable genes. The *E. coli* isolates were submitted to ExPEC PCR testing using the primers presented in Table 2. All PCR products that were amplified with the correct target size (bp) (Table 2) were identified with sequencing and BLAST analysis. Sequencing was done by the Central Analytical Facility of Stellenbosch University and the BigDye™ Terminator V3.1 sequencing kit (Applied Biosystems). The ABI3130xl or ABI3730xl (Applied Biosystems) with a 50 cm capillary array and POP7 (Applied Biosystems) was used for the electrophoresis of the sequences.

RESULTS AND DISCUSSION

Phylogenetic groups (Genotypes)

All the *E. coli* isolates identified with the API 20E system and confirmed with *uidA* PCR (Chapter 3) were tested for their phylogenetic groupings. From the total of seven phylogenetic groups that could be identified with the genotype PCR method (Clermont *et al.*, 2000; Carlos *et al.*, 2010), five were found to be present in the isolates screened in this study. Representations of the Triplex PCR results are given in Fig. 1. Members of phylogenetic group A₀ showed no band, but were regarded as *E. coli* as they were confirmed to be *E. coli* by the presence of the *uidA* gene.

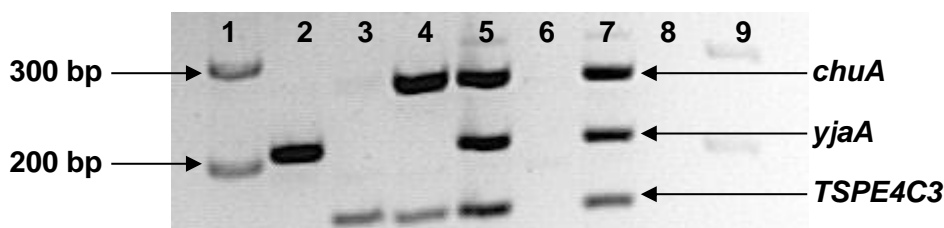


Figure 1 Agarose gel electrophoresis (2% agarose and 1 µg.L⁻¹ ethidium bromide) of a triplex PCR with PCR amplicons. Lanes 1 and 9 = 100 bp marker, lane 8 = negative control, lane 7 = positive control. Lanes 2, 3, 4, 5 and 6 are examples of the phylogenetic groups (lane 2 = A₁; lane 3 = B₁; lane 4 = D₂, lane 5 = B₂₃ and lane 6 = A₀).

The isolates were all grouped into either A₀, A₁, B1, D₂ or B2₃ (Table 3 and Addendum A). No *E. coli* strains belonging to the remaining two groups D₁ and B2₂, were found in this study. The number of isolates of each phylogenetic group as well as the isolation source are listed in Table 3. In total most of the strains (n = 20) had the B1 genotype of which 16 were from farms and four from farmers' markets.

The only phylogenetic group that was isolated from all of the produce sources was A₀ with eight isolates from farms, four from farmers' markets and two from retail outlets. Strains belonging to genotype A are known to mostly consist of commensal strains (Clermont *et al.*, 2000). The same can be said for genotype B1, although INPEC strains according to Pupo *et al.* (1997) can also be found in these groups. Both of these phylogenetic groups were found to be present on produce from farms and farmers' markets. The majority of *E. coli* isolates were sourced from farms and farmers' markets and thus it might be argued that the chance of INPEC strains being present is bigger than ExPEC strains as the majority of isolates were identified as belonging to genotypes A and B1.

Only seven B2₃ isolates were identified and all were sourced from farms. Isolates identified as B2 could also be ExPEC strains (Johnson *et al.*, 2001).

A total of 11 D₂ strains were isolated from farms and farmers' markets. Strains with genotype D could be INPEC strains (Pupo *et al.*, 1997) or ExPEC strains (Johnson *et al.*, 2001).

Duriez *et al.* (2001) reported that in their study the presence of genotypes A and B1 were present the most with 40% and 34%, respectively, and genotypes D and B2 the least present with 15% and 11%, respectively. The study was done on human faeces. These results support the results found in this study with genotypes A (A₁ + A₀), B1, B2 and D being present 40%, 32%, 11% and 17%, respectively. Genotypes A and B1 showed similar results and genotypes B2 and D were in both cases present in the lowest numbers.

The presence of *E. coli* genotype A has frequently been reported in humans and pigs' faecal matter, genotype B1 in non-human mammals, genotype B2 in humans and genotype D in birds and humans (Escobar-Páramo *et al.*, 2006; Carlos *et al.*, 2010; Cook *et al.*, 2011). If these sources are taken into consideration for this study, it can be speculated that the source of the *E. coli* isolates identified as genotype A could originally have come from human or porcine sources. The same can be said for phylogenetic group D, adding the possibility of contamination from birds. The isolates grouped into phylogenetic group B1 were most probably contaminants from non-human mammalian sources like cows, sheep and wild animals. Taking into consideration the variation of phylogenetic groups found to be present in humans (Duriez *et al.*, 2001) it is also possible that the origin of some of the isolates found on the fresh produce in this study could be from human faecal contamination.

It has been reported that *E. coli* are present in soil in subtropical and tropical areas (Ishii *et al.*, 2006). It was also found that the highest loads of *E. coli* in soil were present at higher temperature periods. Their study also indicated that the wet season was during the months with

the lower temperatures and the dry season had the higher temperatures (Ishii *et al.*, 2006). Thus they concluded that *E. coli* is more prevalent during the dry season and warmer months. Furthermore, pathogenic *E. coli* have been shown to survive and increase on produce even at temperatures between 12 and 21°C, but vegetables stored at 5°C and lower showed a decrease in loads (Abdul-Raouf *et al.*, 1993). It is thus clear that *E. coli* prefer warmer temperatures as loads are higher in the dryer and hotter seasons.

The *E. coli* isolates that were sourced in this study were mostly isolated during February which is in the middle of summer in South Africa. There were exceptions of *E. coli* isolated in October and May but these are still not the coldest months in South Africa. It can thus be speculated that this study supports the fact that *E. coli* is more prevalent in warmer temperatures and during the dryer seasons.

Table 3 Number of *E. coli* strains identified in terms of genotypes and source types

Genotypes	Source types			Total
	Farms	Farmers' markets	Retail outlets	
A ₀	8	4	2	14
A ₁	5	6	0	11
B ₁ ₁	16	4	0	20
B ₂ ₃	7	0	0	7
D ₂	3	8	0	11

Fresh produce can be contaminated by various sources. It has been reported that the main sources of contamination are probably irrigation water, human handling and soil. Soil is an indirect contamination source as it is actually more in contact with the root of the plant and internalisation of pathogenic bacteria into the produce can occur during the growth process but at low levels (Deering *et al.*, 2012). Through the soil other factors such as inadequate composted manure can also contribute to contamination (Beuchat & Ryu, 1997; Beuchat, 2002). More direct contamination of fresh produce can occur through contaminated irrigation water as well as handling during harvest, processing and packaging (Brackett, 1999; Beuchat, 2002; Farrar & Guzewich, 2009). The isolates in this study that were sourced from retail outlets could have been contaminated from any contamination source as they were isolated at the end of the production chain. At this stage the produce has undergone the process of harvesting, processing and packaging. The possible contamination sources of the isolates sourced from farmers' markets can possibly be narrowed down to soil transfer, irrigation water and human handling. These products were not put through the whole production process all the way to the retail outlet. The produce sourced directly from the farms was most likely contaminated by irrigation water, soil, animals or birds.

Multiplex PCR

INPEC

Sixty three isolates (Addendum A) were screened with multiplex PCR for intestinal pathogenic *E. coli*. A total of eight genes were tested for. In Fig. 2 all the genes for each INPEC group are presented on an agarose gel.

All the isolates tested positive for the *mdh* household gene which again confirmed their identification as *E. coli*. None of the *E. coli* isolates were found to be positive for any of the INPEC virulence gene sequences, and thus no evidence of risk from INPEC strains was detected.

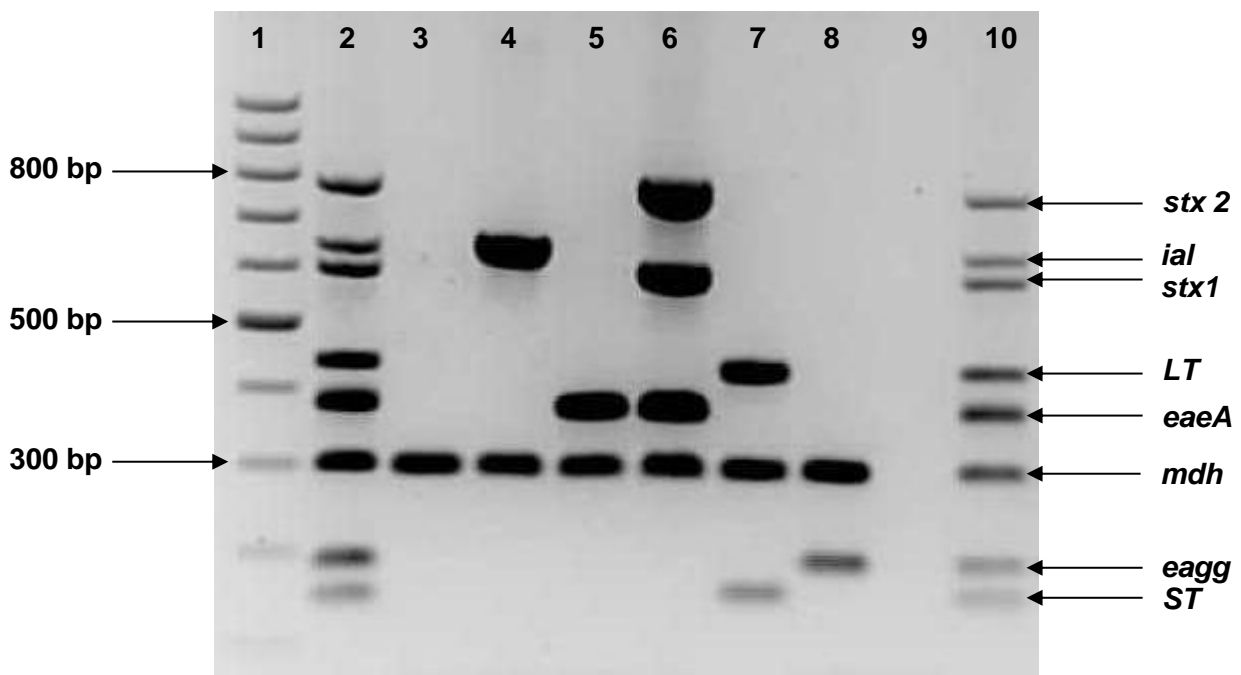


Figure 2 Agarose gel (1.25% agarose and $1 \mu\text{g.L}^{-1}$ ethidium bromide) of INPEC gene sequences amplified with multiplex PCR. Lane 1 = 100 bp marker, lane 2 = positive control, lane 3 = Commensal *E. coli*, lane 4 = EIEC, lane 5 = EPEC, lane 6 = EHEC, lane 7 = ETEC, lane 8 = EAEC, lane 9 = negative control and lane 10 = INPEC gene marker (positive control).

ExPEC

ExPEC multiplex PCR was done on all 63 *E. coli* isolates (Addendum A). The gene sequences tested for with ExPEC PCR are presented in Fig. 3.

Three isolates, 52, 55 and 56, tested positive for the *kpsMT II* gene. The other isolates all tested negative for the ExPEC gene sequences. The *kpsMT II* gene is a group 2 capsular polysaccharide unit (Chapman *et al.*, 2006; Xia *et al.* 2011) giving *E. coli* the ability to synthesise a capsule around its surface which will also indicate the serotype of the *E. coli* strain, e.g. K1 or K12 (Kaper *et al.*, 2004; Bhunia, 2008). This capsule is known to add to bacterial virulence as it enhances survival. The capsule will prevent phagocytosis and thus the bacteria cannot be easily

inactivated in the human body. The presence of the *kpsMT II* gene alone does not imply that these isolates are ExPEC strains as two or more of these genes tested for should be present for an isolate to be classified as an ExPEC strain according to the method of Xia *et al.* (2011). In this study multiplex PCR testing was only done to determine the presence of the six statistically most abundant ExPEC genes (Xia *et al.*, 2011). There are however many other gene sequences that can contribute to ExPEC virulence. Johnson & Stell (2000) reported the presence of 29 different virulence genes that can be found in ExPEC and Chapman *et al.* (2006) reported 36 ExPEC genes that have been identified throughout literature. There could therefore be a possibility that the *kpsMT II* positive strains might be carriers of other ExPEC genes that were not tested for in this study.

According to Johnson *et al.* (2001) it has been shown that *E. coli* strains identified as ExPEC usually belong to phylogenetic group B2 and sometimes D. The three isolates that tested positive for the *kpsMT II* gene were of the phylogenetic group D (Addendum A) which confirmed their probability as ExPEC strains.

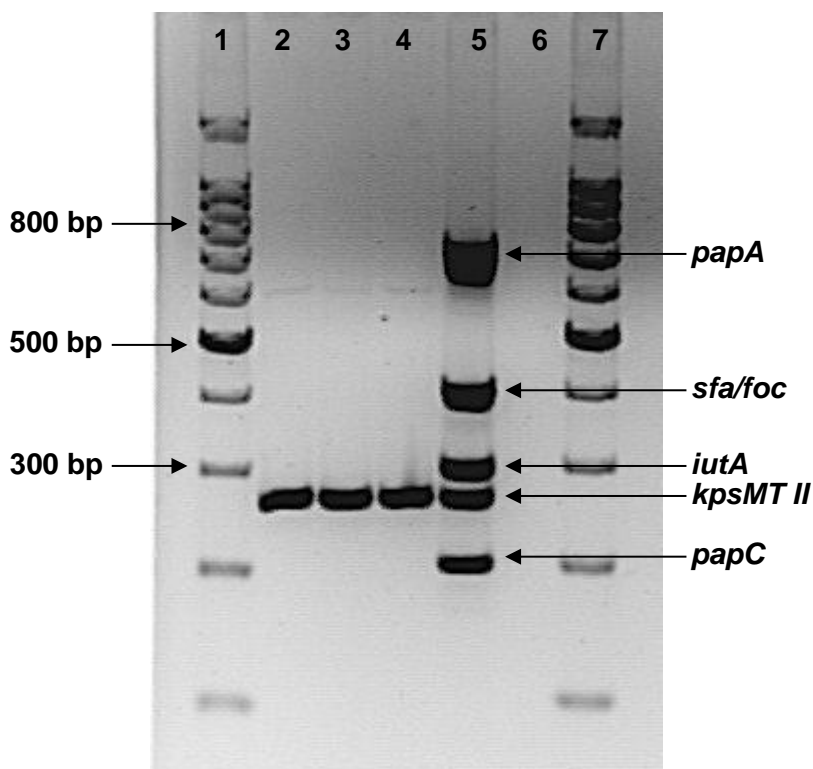


Figure 3 Agarose gel electrophoresis (1.5% agarose and 1 $\mu\text{g}\cdot\text{L}^{-1}$ ethidium bromide) of multiplex PCR testing for extraintestinal pathogenic *E. coli* with PCR amplicons. Lanes 1 and 7 = 100 bp marker, lane 5 = positive control, lane 6 = negative control. Lane 2 = isolate 52, lane 3 = isolate 55 and lane 4 = isolate 56.

Even though only one ExPEC gene was found its presence could qualify the strain as a risk. *Escherichia coli* can easily acquire genes from one another as well as other *Enterobacteriaceae* species through horizontal gene transfer (Karberg *et al.*, 2011). The presence of one gene could therefore indicate that it might have been acquired from a pathogenic strain or that a pathogenic strain could be evolving. A study done by Duriez *et al.* (2001) also found *E. coli* strains that belong to the genogroup B2 that tested positive for only one ExPEC gene and they concluded that these *E. coli* strains seem to be potentially virulent. The latter could also be true for isolates 52, 55 and 56. The indication of a possible pathogenic *E. coli* strain present on produce as found in this study could be considered a risk to the consumer. The data collected was not sufficient to indicate a qualitative risk and thus only a quantitative risk is identified. A quantitative risk is determined based on the “amount” of the data instead of the quality of the data as in the case of a qualitative risk. In future, research should be done to detect the presence of the ExPEC genes for a wider scope of the ExPEC genes and not only the six that were tested for in this study.

All the PCR results (Genogroups, INPEC and ExPEC) for each isolate are presented in Addendum A together with the isolate source and produce type they were isolated from. The three isolates (52, 55, 56) that had the *kpsMT II* gene were sampled on the same day and they are all from the same produce source, parsley. The possibility that they can all be clones was considered but isolate 52 however had a different API profile than strains 55 and 56. It tested negative for biochemical characteristics ONPG, RHA and MOB which is in contrast to the results obtained for isolates 55 and 56. Isolate 52 thus differs from isolates 55 and 56.

Phenotypic characteristics compared with phylogenetic characteristics

In Chapter 3 of this thesis dendrograms of all the isolates were constructed and discussed in terms of similarity clusters based on their phenotypic characteristics. In this chapter the S_{SM} coefficient dendrogram is again presented to be discussed in terms of phylogenetic groupings found within the clusters of the dendrogram (Fig. 4). This was done as it could be argued that genotypes could be linked to clusters that were formed based on phenotypic characters.

The dendrogram shows four *E. coli* clusters (C, G, H and I) and thus these are discussed. All the isolates that grouped in Cluster C were sampled from beans sourced from both farms and retail outlets. Most of these isolates (81, 69, 73 and 7) were identified as genotypes A_1 or A_0 (Addendum A) except for isolates 50 and 51 that could not be identified as *E. coli*.

All the isolates (80, 82, 63, 78, 76, 75, 74, 71, 70, 68, 67, 66, 61, 62, 64 and 65) grouped in Cluster G were only sourced from beans. These beans had been irrigated with water from the Plankenburg River, and varied in their genotypes (B_1 , A_1 , B_2 and A_0) (Addendum A).

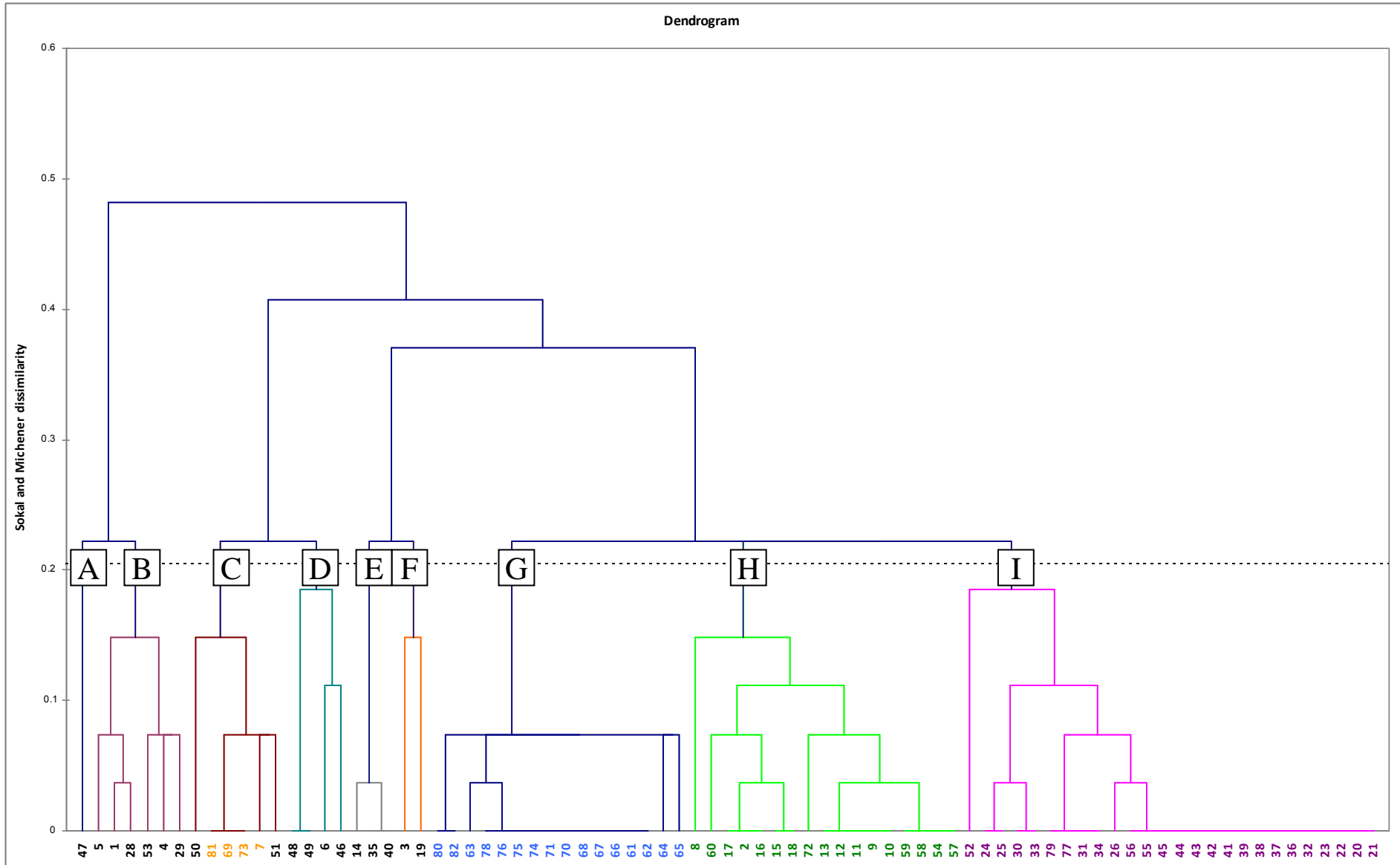


Figure 4 Dendrogram of all 81 isolates excluding ATCC reference strains done with the S_{SM} coefficient method. Cluster H consist of isolates all identified as genogroup A and Cluster I consist of isolates identified as either genogroup B1 or D.

The isolates (8, 60, 17, 2, 16, 15, 18, 72, 13, 12, 11, 9, 10, 59, 58, 54 and 57) grouped in Cluster H were all identified as phylogenetic group A₁ or A₀ (Addendum A). The isolates (52, 24, 25, 30, 33, 79, 77, 31, 34, 26, 56, 55, 45, 45, 43, 42, 41, 39, 38, 37, 36, 32, 23, 22, 20 and 21) in Cluster I were all identified as either B1 or D with one exception, isolate 77, which was B2₃ (Addendum A).

When looking at Clusters C and H it can be concluded that there is a correlation between the isolates' phenotypic and phylogenetic characteristics as the isolates in these Clusters had the same genotypes, A₁ and A₀. All of the *E. coli* isolates contributing to Clusters C and H were sampled from different sources (farms, retail outlets, farmers' markets) and different produce types (beans, peas, parsley). Taking into consideration clusters G and I where the isolates belong to a combination of phylogenetic groups and were isolated from a variety of sources, it can be concluded that there are a variety of *E. coli* present on the produce surfaces. Leff & Fierer (2013) also reported that there were a wide variety of bacteria present on the surface of produce and also that in their study they differed between produce types. This supports the variety of *E. coli* found on different types of produce in this study.

CONCLUSION

The isolates sampled in this study were phylogenetically grouped into five of the eight groups from which group A₀ was the only one that was identified on produce from all of the source types. This is an indication that there is a risk of the presence of *E. coli* on produce from different sources. This could also be a positive observation as no specific source type can be linked to *E. coli* presence on produce and thus decrease the possibility of a localised disease outbreak. Phylogenetic group B1 was the most common (20 isolates) followed by 14 A₀ isolates, 11 isolates each for A₁ and D₂ and seven isolates for genotype B2₃. The presence of a variety of genogroups indicates that the original sources of pollution could be multiple.

None of the isolates in this study was positive for any INPEC gene sequences and thus no INPEC strains were identified. The latter is beneficial for the food industry and ultimately for the consumer as it indicates a reduced risk of pathogens being present.

Only three of the isolates in this study tested positive for one ExPEC gene, *kpsMT II*, and none of the other ExPEC genes tested for with Multiplex PCR were found to be present. These three isolates cannot be classified as ExPEC strains but it is recommended that further testing for other ExPEC gene sequences be done in the future. The fact that the *kpsMT II* gene was present indicates that there is a risk of pathogenic *E. coli* evolving or multiplying. *Escherichia coli* also have the ability to acquire genes horizontally from other *Enterobacteriaceae* species and this can also add to new pathogenic strains emerging.

Most of the *E. coli* isolates that were found present on the produce were not identified as pathogenic strains and this is to the advantage of the consumer. These include all produce

samples tested from farmers' markets and retail outlets. An indication of a risk for possible ExPEC strains was however found on parsley samples sourced from a small-scale farm.

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Addendum A*Escherichia coli* isolates, their isolation source and PCR (Triplex and multiplex) results

Isolate	Source	Produce	Genogroup	INPEC	ExPEC
2	Retail 3	Beans	A ₀	-	-
7	Retail 3	Beans	A ₀	-	-
8	Market 1	Peas	A ₁	-	-
9	Market 1	Peas	A ₁	-	-
10	Market 1	Peas	A ₁	-	-
11	Market 1	Peas	A ₁	-	-
12	Market 1	Peas	A ₁	-	-
13	Market 1	Peas	A ₁	-	-
15	Market 3	Peas	A ₀	-	-
16	Market 3	Peas	A ₀	-	-
17	Market 3	Peas	A ₀	-	-
18	Market 3	Peas	A ₀	-	-
20	Farm 2	Cabbage	B1	-	-
21	Farm 2	Cabbage	B1	-	-
22	Farm 2	Cabbage	B1	-	-
23	Farm 2	Cabbage	B1	-	-
24	Market 3	Beans	D ₂	-	-
25	Market 3	Beans	D ₂	-	-
26	Market 6	Lettuce	B1	-	-
30	Farm 3	Cabbage	B1	-	-
31	Farm 3	Cabbage	B1	-	-
32	Farm 3	Cabbage	B1	-	-
33	Farm 3	Cabbage	B1	-	-
34	Farm 3	Cabbage	B1	-	-
36	Market 2	Spinach	D ₂	-	-
37	Market 2	Spinach	D ₂	-	-
38	Market 2	Spinach	D ₂	-	-
39	Market 2	Spinach	D ₂	-	-
41	Market 2	Spinach	D ₂	-	-
42	Market 2	Spinach	D ₂	-	-
43	Market 6	Lettuce	B1	-	-
44	Market 6	Lettuce	B1	-	-
45	Market 6	Lettuce	B1	-	-
52	Farm 5	Parsley	D ₂	-	<i>kpsMT II</i>
54	Farm 5	Parsley	A ₀	-	-
55	Farm 5	Parsley	D ₂	-	<i>kpsMT II</i>

Isolate	Source	Produce	Genogroup	INPEC	ExPEC
56	Farm 5	Parsley	D ₂	-	<i>kpsMT II</i>
57	Farm 5	Parsley	A ₀	-	-
58	Farm 5	Parsley	A ₀	-	-
59	Farm 5	Parsley	A ₀	-	-
60	Farm 5	Parsley	A ₀	-	-
61	Farm	Beans	B1	-	-
62	Farm	Beans	B1	-	-
63	Farm	Beans	A ₁	-	-
64	Farm	Beans	A ₁	-	-
65	Farm	Beans	A ₁	-	-
66	Farm	Beans	A ₀	-	-
67	Farm	Beans	B2 ₃	-	-
68	Farm	Beans	B1	-	-
69	Farm	Beans	A ₀	-	-
70	Farm	Beans	B1	-	-
71	Farm	Beans	B2 ₃	-	-
72	Farm	Beans	A ₁	-	-
73	Farm	Beans	A ₀	-	-
74	Farm	Beans	B2 ₃	-	-
75	Farm	Beans	B2 ₃	-	-
76	Farm	Beans	B2 ₃	-	-
77	Farm	Beans	B2 ₃	-	-
78	Farm	Beans	B2 ₃	-	-
79	Farm	Beans	B1	-	-
80	Farm	Beans	B1	-	-
81	Farm	Beans	A ₁	-	-
82	Farm	Beans	B1	-	-

- = Not detected

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Fresh produce is considered worldwide to be a vital component of a healthy balanced diet and it is also a relatively economical food source. It is thus important to ensure that the fresh produce is safe for the consumer. South Africa is a water scarce country with the farmers using river water for irrigation of produce. These water sources are known to be heavily polluted.

Foodborne outbreaks linked to fresh produce are increasing world-wide and are thus a growing concern. This will not only be detrimental to the consumer but also to the food industry as sales will be impacted if fresh produce is unsafe to consume. The latter can ultimately negatively influence the economy of South Africa as well. It is thus clear that the safety of fresh produce should be ensured before sale and consumption.

The overall objective of this study was to determine the coliforms contamination loads of selected production sites and more specifically the *E. coli* loads on fresh produce. Additionally the specific *E. coli* types present on fresh produce were evaluated. This was done by sampling fresh produce from “point-of-harvest” (PoH) and “post-harvest” (Ph) sample sites in the Western Cape. A wide variety of fresh produce from different source types and produce types was sampled and tested. Isolated *E. coli* were categorised into phenotypes, genotypes and pathotypes (INPEC, ExPEC), if present. In addition a brief risk assessment was done to evaluate the potential hazard of pathogenic *E. coli* on fresh produce. This was done in terms of source type, produce type and frequency.

High total coliforms (TC) loads were found on most of the produce and *E. coli* was present on 8% of the produce samples. The highest TC load found on the fresh produce was log 8.38 MPN.100 mL⁻¹ TC on lettuce, tomatoes, cabbage and spinach and the highest *E. coli* load found was log 7.38 MPN.100 mL⁻¹ *E. coli* on cabbage. In terms of the TC loads on the fresh produce the majority of samples exceeded the recommended guidelines (<200 cfu.g⁻¹ TC = <15 000 cfu.100 mL⁻¹ TC) of the South African Department of Health (DoH, 2011). The data showed that the presence of TC did not always indicate the presence of *E. coli*. The coliform group do, however, include a number of other faecal pathogens, so TC can still cause illness if sufficient numbers are ingested (DAAF, 1996).

It was found that *E. coli* was most common on produce from farmers' markets and the lowest on produce from retail outlets with the highest log 8.38 MPN.100 mL⁻¹ *E. coli* on cabbage sampled from a commercial farm. It was concluded that the produce from farmers' markets are the contaminated with the highest loads of both TC and *E. coli*. It could be that the produce at farmers' markets are harvested from small-scale farms that use polluted water for irrigation or are using improperly digested manure as compost or are using a section of land close to where animals are grazing or are simply not following sufficient safety precautions during the production process.

The results found do not indicate a clear pattern in terms of *E. coli* being present on a certain produce type or source type. Thus the presence of *E. coli* will not be predictable according to results found in this study based on produce type and source type. The only hint of a prediction that can be made is that *E. coli* might be present in higher loads in the warmer months since *E. coli* was mostly detected during the warmer months in this study.

The *E. coli* numbers found all exceeded the recommended guidelines for raw vegetables according to the South African Department of Health (DoH, 2011) ($<0 \text{ cfu.}100 \text{ mL}^{-1} \text{ E. coli}$). In total *E. coli* was found present on 12 out of 151 produce samples from all of the sources and thus *E. coli* was present on 8% of the fresh produce samples. In terms of food safety *E. coli* should be absent on produce and thus the 8% *E. coli* present is unacceptable. The risk of produce being contaminated with *E. coli*, pathogenic or commensal, should be of concern to both produce farmers, local authorities and the food industry. Based on the results in this study, the biggest risk in terms of source type is farmers' markets as 4 of the 8% *E. coli* detected in this study was on produce from farmers' markets and the remaining 4% was on produce from retail outlets and farms. The reason for farmers' markets presenting the biggest risk is unsure but as mentioned above, it is possible that the produce could be from small-scale farms where produce could be exposed to various contamination sources and unhygienic practices. Consumers should be aware of this risk and wash or sanitise the produce thoroughly before consumption. This will not always be efficient if pathogenic *E. coli* are present as some of the pathogenic *E. coli* produce heat labile toxins or it could have attachment characteristics that contribute to the bacteria's virulence and has low infective doses.

In total 63 *E. coli* strains were isolated and grouped in three main phenotypic dendrogram clusters. The data clearly showed that there is a wide range of *E. coli* types present in terms of phenotypic characteristics. The latter indicates that these different *E. coli* clusters have different characteristics and thus they will probably function differently at a biochemical level. What was interesting was that the members of these three groups could not, however, be assigned to specific produce types or produce sources in this study.

The 63 *E. coli* isolates were then further subjected to triplex PCR to categorise the *E. coli* isolates to their various genotypes, and secondly to multiplex PCR (INPEC and ExPEC) to determine the presence of pathogenic *E. coli*. All four of the main genotypes (A, B1, B2 and D) were found present and five of the seven sub-groups (A_0 , A_1 , B1, $B2_2$, $B2_3$, D_1 and D_2) were present. The genotype distribution indicates again that a wide variety of *E. coli* is present on fresh produce. Ultimately, this can be harmful to the consumer's health as it cannot be predicted which specific *E. coli* type is present on a specific produce type or if a specific *E. coli* type will be present on produce from a certain source type. In this case it is nearly impossible to control an unpredictable risk but preventative measures can be and should be implemented. The latter should include safety precautions at every stage on the farm from pre-harvest to the consumer's kitchen. Safety precautions should include monitoring the microbial levels of produce, taking

preventative measures in terms of hygiene and elimination if any hazard is present. This means that a secure food safety system should be implemented. The unpredictability of a specific *E. coli* type on a specific produce or from a certain source type can also be observed as decreasing the risk of a localised disease outbreak, as no clear contamination source could be documented.

In this study the presence of only one ExPEC gene, *kpsMT II*, was detected in three isolates, 52, 55 and 56. The *kpsMT II* gene is a group 2 capsular polysaccharide unit giving *E. coli* the ability to synthesise a capsule around its surface which is known to add to the bacteria's virulence as it enhances the bacteria's survival. Phagocytosis will be prevented by this capsule and thus the bacteria cannot be easily inactivated in the human body. Six ExPEC genes were tested for from which two or more should be present for *E. coli* to be classified as an ExPEC. None of the *E. coli* isolates in this study had more than one ExPEC gene present. The mere presence of a pathogenic gene could indicate that other pathogenic genes, which were not tested for, could also be present. *Escherichia coli*, as well as the rest of the *Enterobacteriaceae* family, are able to easily transfer genes horizontally. This thus increases the possibility of pathogenic bacteria (*E. coli* and other) to evolve in the environment (water and soil), even if pollution is not occurring. This increases the risk of pathogenic *E. coli* occurring on fresh produce.

Thus the conclusion made in this study is that *E. coli* is present on fresh produce but the location, loads, types and extent thereof is unpredictable. Contaminated fresh produce will also have a negative effect on the food industry as it will reduce sales and ultimately reduce employment which is already a problem in South Africa. The unpredictable risk of potentially contaminated produce can have a negative impact on the consumer, the food industry and the South African economy.

More strict regulations in terms of microbiological quality of fresh produce should be put into place and made compulsory to be implemented in the fresh produce production chain. The only produce specific guidelines in South Africa are recommended guidelines from the Department of Health. Farmers and producers should also be made aware of the risk and they should implement good agricultural practices (GAP) and a quality control system such as HACCP (Hazard Analysis Critical Control Point) to ensure the safety of the produce at every stage during harvest through to the consumer. It is important to know when and how contamination occurs. The latter will be achieved with a quality control system in place throughout the harvest and production chain. Should the produce be contaminated at any stage, precautions must be taken to eliminate or reduce the risk to an acceptable level, before it goes to the next stage. It is also recommended that an in-depth study be done, to look at what the environment should look like for certain *E. coli* types to survive and multiply in terms of water activity, pH and nutrients and compare this with the surface environment of specific produce types. This should give a better indication of where *E. coli* will be more prone to be present in terms of produce type and source type.

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