Bacteriological quality of South African irrigation water and its role as a source of contamination on irrigated lettuce

By

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DECLARATION

I declare that the dissertation herewith submitted for the degree MSc Food Science at the University of Pretoria has not previously been submitted by me for a degree at any other university or institution of higher learning.

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DEDICATION

This work is dedicated to my aunt Dr. Edrone Rwakaikara and her family.

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ABSTRACT

BACTERIOLOGICAL QUALITY OF SOUTH AFRICAN IRRIGATION WATER AND ITS ROLE AS A SOURCE OF CONTAMINATION ON IRRIGATED LETTUCE

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A deteriorating trend has been noted in the bacteriological quality of surface irrigation water sources in South Africa. In a bid to compare the bacteriological quality of two irrigation water sources as well as whether irrigation water was a source of bacterial pathogens on irrigated lettuce, this study was designed and divided into two phases. Phase one involved determination of physico-chemical parameters and bacterial indicators in the Loskop canal, the Skeerpoort river and lettuce irrigated with water from the Skeerpoort river over 10 months. Co-currently the study further determined the diversity of the most prevalent bacterial microflora in the 3 sample sources over the same time period.

Aerobic colony counts (ACC), Aerobic spore formers (ASF), Anaerobic spore formers (AnSF), Faecal coliforms (FC), Intestinal enterococci (IE) and *Staphylococcus aureus* (*S. aureus*) as well as prevalence of *Escherichia coli (E. coli*), *Salmonella* spp and *Listeria monocytogenes* (*L. monocytogenes*) were determined. Additionally the most prevalent aerobic bacterial species isolated from the three sources were determined. Higher mean rainfall was noted in areas surrounding the Skeerpoort river (74.7mm) than the Loskop canal (0.1mm). Mean temperature was 15.4˚C and 18.2˚C while mean pH was 7.4 and 8.4 in the Loskop canal and the Skeerpoort river respectively. Low mean bacterial counts of less than 3.4 $\log_{10}$cfu/ml, were noted for ACC, ASF, AnSF, *S. aureus* and IE at both irrigation sites. Higher mean ACC of 5.9 $\log_{10}$cfu/g and *S. aureus* counts of 3.0 $\log_{10}$cfu/g were noted on

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lettuce. Although low mean counts of FC (1.3 $\log_{10}$cfu/100ml) were noted for all three sources, high incidence of *E. coli* was observed during bacterial composition studies on non-selective media. This suggested underestimation of faecal contamination possibly indicating that identification of specific pathogens provided a better measure of assessing bacterial contamination than bacterial indicators. *E. coli*, *Bacillus* spp and *Enterobacter* spp were the most prevalent bacteria in the Loskop canal, the Skeerpoort river and on lettuce. Prevalence of *E. coli*, *Bacillus* spp and *Enterobacter* spp in the Loskop canal was 23%, 33% and 26% respectively. Similarly prevalence in the Skeerpoort river was 36%, 26%, 16% respectively. On lettuce prevalence of the same bacteria was 36%, 30% and 6% respectively. *E. coli* O157:H7 was isolated at both irrigation sites while *Salmonella enterica* (gp 1) ST paratyphi A was isolated from the Skeerpoort river. High prevalence of similar bacterial species within the Loskop canal and the Skeerpoort river suggested similar sources of contamination in the two water sources inspite of different geographical location and surrounding land use practices. Additionally, similar bacterial species in irrigation water from the Skeerpoort river and on irrigated lettuce suggested water as a source of contamination on produce. Additionally it suggests ability of bacterial pathogens to withstand environmental conditions under field conditions which may pose a risk to food safety and public health among individuals consuming irrigated fresh produce.

Phase 2 aimed at determining the prevalence of antibiotic resistant and virulent *E. coli* collected from the Loskop canal, the Skeerpoort river and lettuce irrigated with water from the Skeerpoort river. Forty one (41) *E. coli* isolates: (19) Loskop canal; (12) the Skeerpoort river; (10) lettuce were tested with 11 antibiotics at single concentrations and screened for Shigatoxin 1 (*stx 1*), Shigatoxin 2 (*stx 2*) and intimin (*eae*) genes. Antibiotic resistance was also used as a means of clustering *E. coli* isolated from the 3 sources. In the Loskop canal 84% and 83% of strains in the Skeerpoort river were resistant to at least one antibiotic. There was a significant difference ($p \leq 0.05$) in resistance to antibiotics between isolates from the Loskop canal and the Skeerpoort river. Additionally the combined effect of isolate source (irrigation water site) and antibiotics for isolates from the Skeerpoort river was significant ($p \leq 0.05$). From lettuce, 90% of isolates were resistant to at least one antibiotic and resistance significantly differed ($p \leq 0.05$) from isolates in the Skeerpoort river. The highest resistance to single antibiotics in all three samples was to cephalothin and ampicillin. Higher resistance was noted to multiple (more than 2) antibiotics in the Skeerpoort river (33%) than Loskop canal (5%). Most isolates from the same source showed close relatedness.

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Close relatedness was noted between isolates from the Loksop canal (10.5%) and the Skeerpoort river (16%). From irrigated lettuce 40% of isolates showed close relatedness to isolates in irrigation water from the Skeerpoort river. In the Loskop canal 15% and 41% of isolates in the Skeerpoort river possessed virulence genes. From lettuce, 20% of isolates possessed virulence genes. In the Loskop canal as well as from lettuce all isolates with virulence genes were antibiotic resistant while 80% of isolates with virulence genes in the Skeerpoort river were antibiotic resistant. In the Loskop canal 10% and 25% of isolates in the Skeerpoort river were positive for *stx1*/*stx2* and *eae*, genes synonymous with Enterohaemorrhagic *E. coli* (EHEC). Results from this study show that *E. coli* from the two irrigation water sources as well as on irrigated lettuce were resistant to antibiotics and potentially pathogenic. This may increase risk of contaminating irrigated fresh produce which may compromise food safety and public health of consumers.

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CHAPTER 1: PROBLEM STATEMENT

Surface water sources such as rivers and canals provide the bulk of irrigation water used for agriculture in South Africa (Center for Scientific Research and Innovation (CSIR), 2010). These water sources flow through vast areas of the country providing water for other sectors such as industry, mining and domestic use. This may strain water sources as all sectors try to maintain growth. Additionally such a scenario exposes surface water sources to increased risk of contamination with pathogenic bacteria. A number of studies carried out to determine the bacteriological quality of irrigation water in South Africa have reported on its deteriorating quality (Obi *et al*., 2004; Kinge *et al*., 2010; Ijabadeniyi *et al*., 2011b; Gemmel and Schmidt, 2012). Factors such as an aging sewage treatment infrastructure, illicit disposal of industrial waste and informal settlements have been indicated as possible sources of contamination (Britz *et al*., 2012). Therefore use of such water for irrigating fresh produce may present risk of pathogenic bacterial contamination as well as foodborne illness to consumers in case post-harvest processing fails to eliminate all the pathogens.

South Africa is a major producer and exporter of fresh produce grown under irrigation therefore ensuring the bacteriological quality of such water is essential in ensuring production of safe food and maintaining a lucrative industry. Irrigation water has been noted as a source of bacterial contamination on irrigated fresh produce (Islam *et al*., 2004; Ijabadeniyi *et al*., 2011b; Gemmel and Schmidt, 2012). The Food and Drug Administration (FDA) estimates approximately 48 million cases of foodborne illnesses occur in the United States alone (FDA, 2013). Additionally fresh produce has been implicated in a number of foodborne illnesses around the world (Doyle and Erickson, 2008). Bacterial pathogens have been found on different vegetables such as cabbage and lettuce (Harris *et al*., 2003; Lötter, 2010). Enteric pathogens such as *Escherichia coli* O157: H7, *Salmonella* species and *Listeria monocytogenes* which are usually of faecal origin pose the most serious food safety concerns (Ampadu, 2007; Kreske, 2009; Olaniran *et al*., 2009) because they cause food borne-illnesses (Ijabadeniyi *et al*., 2011a).

The worldwide increase in foodborne outbreaks associated with fresh produce many of which have led to a number of fatalities has increased awareness of safety among stakeholders in the food industry (Doyle and Erickson, 2008). The outbreak of *E. coli* O104:H4 in Germany previously not linked to fresh produce (Rasko *et al*., 2011) as well as *L. monocytogenes* and

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E. coli O157 in the United States (Centers for Disease Control and prevention (CDC), 2011) depicts the risks to food safety and public health posed by bacterial foodborne pathogens. The occurrence of a number these recent high profile foodborne outbreaks in the developed world in spite of stringent quality control measures, suggests that developing countries such as South Africa may face similar risks. Therefore there is a need for measures to limit such outbreaks which ultimately depends on reliable information relating to prevalence, diversity and virulence of bacterial foodborne pathogens.

Although some studies in South Africa have reported on the bacteriological quality of irrigation water sources, they focused on the prevalence and characterization of bacterial pathogens (Ackermann, 2010; Lötter, 2010; Duhain, 2011; Ijabadeniyi *et al*., 2011b). In order to reliably assess the level of bacterial contamination, there is need to assess the composition and diversity of bacteria within individual sources (Sapers and Doyle, 2009). Additionally there is need to characterize pathogens in order to determine the immediate risk posed to causing illness in consumers. Since most fresh produce undergoes minimal postharvest processing, pathogens such as *E. coli* O157: H7 with a low infectious dose may survive causing disease in the final consumer. For example Obi *et al*., (2004) and Olaniran *et al*., (2009) used both antibiotic resistance and screening of virulence genes in *E. coli* to assess the contamination levels in two South African water sources. By using antibiotic resistance, inference on probable sources of bacterial contamination may be determined helping to provide more reliable information that could be used in mitigating pollution.

This study aimed at comparing pathogenic bacterial contamination in two irrigation water sources the Loskop canal and the Skeerpoort river located within two provinces of South Africa, Mpumalanga Province and North West Province respectively. Additionally the study aimed at determining whether irrigation water from the Skeerpoort river was a source of bacterial contamination on irrigated lettuce grown under field conditions. In both phases of the study, the diversity of bacteria as well as prevalence of antibiotic resistant and virulence genes in *E. coli* was determined in a bid to provide more reliable information relating to the risk posed to public health and food safety.

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CHAPTER 2: LITERATURE REVIEW

2.1 WATER SITUATION IN SOUTH AFRICA

Natural fresh water sources such as rivers provide water used for many applications such as farming, cleanliness and sanitation, environment maintenance and economic development. South Africa is a semi-arid country where water is of critical strategic importance to all development in any sector of the economy (Department of Water Affairs (DWAF), 2008). Water allocations in the country are shown in Table 1. The National Water Act (NWA) (Act 36 of 1998) provides for water to be protected, utilized, developed, conserved, managed and controlled in a sustainable and equitable manner (DWAF, 2008).

Table 1: Water resource allocations in South Africa as of 2008 per water user group

Water user/sector	Proportion of allocation (%)
Agriculture	62
Domestic	27
Urban	23
Rural	4
Industrial	3.5
Afforestation	3.0
Mining	2.5
Power generation	2.0

(Adapted from DWAF, 2008)

Water use in South Africa is dominated by irrigation which accounts for approximately 62% of all water used in the country (CSIR, 2010). There is concern about the declining quality of water in rivers and dams as a result of pollution and land use management which has social, economic and environmental implications (DWAF, 2008). Sources of pollution include agriculture and industrial activities, poorly managed waste management treatment works and human settlements (DWAF, 2008). Of concern for human and ecosystem health is occurrence, transport and fate of disease-causing microorganisms in the aquatic environment.

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The deteriorating quality of irrigation water in South African water sources has led to initiatives spear-headed by The Water Research Commission (WRC) and Department of Agriculture, Forestry and Fisheries (DAFF). Under a previous project (K5/1773) also funded by the WRC and co-funded by DAFF with the theme 'quantitative investigation into the link between irrigation water quality and food safety', studies were carried out which reported on the deteriorating bacterial quality in several irrigation water sources in South Africa. These water sources were located across a number of provinces such as; Western Cape (Ackermann, 2010; Lötter, 2010), KwaZulu-Natal (Gemmel and Schmidt, 2012), North West (Duhain, 2011) and Mpumalanga (Ijabadeniyi *et al*., 2011b).

Irrigation water with poor bacteriological quality may have negative effects down-stream the processing chain such as health inspectors rejecting fresh produce export because of bacterial contamination (CSIR, 2010). The overall, water quality will progressively deteriorate unless corrective management actions are implemented effectively and continuously (CSIR, 2010). According to the CSIR "All life is dependent directly or indirectly on the healthy functioning of the aquatic eco-system. Globally, unsafe water, inadequate sanitation and poor hygiene are rated among the top 10 risks to health." (CSIR, 2010). The project K5/1875/4 under which the present study falls was initiated with the theme named 'An investigation into the link between water quality and microbiological safety of fruit and vegetables from the farming to the processing stages of production and marketing.'

2.2 VEGETABLE GROWING IN SOUTH AFRICA

The agro-processing industry which is dependent on irrigation makes up 20% of South Africa's Gross Domestic Product (GDP) (DAFF, 2012). Additionally it is an important source of foreign exchange earnings as well as a crucial source of employment especially in rural areas, employing 15% of the labour force (DAFF, 2012). Vegetables grown in South Africa include among others; lettuce, tomatoes, cabbage, butternut crush generating revenue of approximately R 5.5 billion (DAFF, 2012). A large proportion of the vegetables grown are exported to the United States and the European Union (Britz *et al*., 2012).

There has been a worldwide increase in demand for fresh produce such as vegetables (Aruscavage, 2007). This has been due to health benefits associated with eating vegetables such as provision of vitamins, minerals and phyto-chemicals which improve health and well-being. At least 5 to 13 servings of fruit and vegetables a day have been recommended for

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healthy well-being and their convenience as ready-to-eat food stuffs has made them popular among the public (Ampadu, 2007).

The growing and consumption of vegetables is being encouraged in South Africa to boost health within the population (Britz *et al*., 2012). Growing vegetables requires water from rivers for irrigation. However the rising population density puts strain on rivers and other open water sources increasing their risk to pathogenic bacterial contamination (CSIR, 2010). This is because irrigation especially in developing countries is frequently carried out using untreated waste water which might contaminate fresh produce such as vegetables with bacterial pathogens (Al-sa´ed, 2007). Therefore there is a need to evaluate the bacteriological quality of vegetables grown under irrigation water from river sources whose water has previously shown high prevalence of bacterial pathogens.

2.3 FOODBORNE BACTERIAL PATHOGENS

Over the last 40 years an increasing trend of foodborne outbreaks caused by bacterial pathogens has occurred with about half of them originating from zoonotic sources (Newell *et al*., 2010). Policy makers such as those in government and research institutions focus on pathogens based on; predominance of outbreaks, severity of disease outcomes and public interest (Newell *et al*., 2010). Therefore focus on foodborne pathogens is majorly concerned with those previously implicated in widespread human illnesses.

Common foodborne bacterial pathogens associated with fresh produce include; *Salmonella* spp, *E. coli*, *Shigella* spp, *L. monocytogenes* and *Bacillus cereus* (Olaimat and Holley, 2009; Pachepsky *et al*., 2011; Faruque, 2012). Other pathogens associated with fresh produce include *Campylobacter* spp and *Staphylococcus* spp (Whipps *et al*., 2008; Newell *et al*., 2010). However, of all these pathogens, those most commonly associated with fresh produce related outbreaks include; *Salmonella* spp, Enterohemorrhagic *E. coli* (EHEC) O157:H7, Enterotoxigenic *B. cereus* and Enterotoxigenic *Staphylococcus aureus* (Pachepsky *et al*., 2011). Investigative studies carried out in South Africa to determine the prevalence of bacterial foodborne pathogens in irrigation water and on irrigated fresh produce have reported presence of *E. coli*, *Salmonella* spp and *L. monocytogenes* (Ijabadeniyi *et al*., 2011b; Duhain, 2011; Ackermann, 2010; Lötter, 2010). Additionally studies carried out in other parts of the country to determine prevalence of bacterial foodborne pathogens in rivers have reported high prevalence of bacterial pathogens such as *E. coli* (Obi *et al*., 2002; Olaniran *et al*., 2009) indicating that surface irrigation water sources may have the potential for

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contaminating irrigated fresh produce. Therefore this review will focus on bacterial foodborne pathogens that have previously been implicated in foodborne illnesses associated with fresh produce around the world.

2.3.1 *ESCHERICHIA COLI* (*E. COLI*)

E. coli is commonly used as an indicator of faecal pollution from human and animal sources. There are 5 groups of *E. coli* (diarrhoeagenic) types that have been linked to foodborne outbreaks (Faruque, 2012). These strains have evolved from non-pathogenic to pathogenic strains through acquisition of mobile genetic elements such as; colonising factors, enterotoxins, cytotoxins, haemolysins and invasins (Faruque, 2012). The ability of human *E. coli* isolates to colonise cattle may in part explain its emergence as an important human pathogen (Law, 2000). The colonisation of cattle and then contamination of bovine products (beef and milk) has allowed the organism to enter the human food chain (Law, 2000).

PATHOGENIC STRAINS OF *E. COLI*

According to Law (2000), there are two mechanisms through which non-pathogenic bacteria become pathogenic. Firstly, divergence and vertical transmission of existing genes by mutations, recombination and rearrangement. Secondly horizontal exchange of information between bacteria of the same and different species can also occur. For horizontal gene exchange to be effective, the acquired gene(s) should confer a competitive advantage to the recipient microorganism (Law, 2000).

During horizontal exchange, Pathogenicity Islands (PI's), encode functions such as adhesins or an iron uptake system assisting bacteria to adapt to new environments (Law, 2000). Pathogenic strains of *E. coli* include; Enteroheamorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAEC) and Enteroinvasive *E. coli* (EIEC). However foodborne outbreaks are more associated with EHEC which produces shigatoxin or verotoxin ultimately causing acute gastroenteritis, heamorrhagic colitis (HC) and heamorrhagic uremic syndrome (HUS) (Newell *et al*., 2010). Numbers of *E. coli* on produce should not be higher than 10^3 cfu per 25 g of product and pathogenic *E. coli* should be absent in 25 g of product (Weiss *et al*., 2011).

The pathogenicity of EHEC has been associated with the following virulence factors; shigatoxins, adherence factors, locus of enterocyte effacement (LEE) and intimin (Welch, 2006). Shigatoxins are characterized into shigatoxin 1 (*Stx 1*) and shigatoxin 2 (*Stx 2*). The adherence factors on EHEC help it attach to sites within hosts such as small intestines. The

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toxins and adherence factors are usually coded by genes located on specific regions of the bacterial genome called pathogenicity islands such as LEE (Welch, 2006). The pathogenicity of EHEC in humans occurs through development of attaching and effacing lesions in the gut, local arrangement of the host cell cytoskeletal of epithelial cells and secretion of electrolyte and liquid into the gut (Weiss *et al*., 2011).

Diagnosis of EHEC relies on detection of specific antigen O157 or H7 and virulence factors *Stx 1*, *Stx 2* and intimin (*eae*) (Faruque, 2012). Geographical differences in both prevalence and serotype distribution in all verotoxin producing *E. coli* (VTEC) types suggest that some countries show host specificity possibly as a result of husbandry factors and restricted exposure (Newell *et al*., 2010). In the United States, Japan and Great Britain the serotype O157: H7 is most prevalent while in other countries serotypes such as O26 and O111 have been reported to cause disease (Welch, 2006). *E. coli* O157: H7 isolated from Australia was found to have lower virulence compared to those in other countries (Law, 2000).

E. coli O157:H7 has been frequently linked to fresh produce such as spinach and lettuce on which it poses a major food safety risk, increasing the likelihood for illness (Olaimat and Holley, 2009). In a study to characterize virulence genes in *E. coli* O157:H7 isolated from various samples in The North West Province of South Africa, Ateba and Mbwe (2011), noted *eae* and *hylA* (enterohemolysin gene A) genes in *E. coli* isolated from a river catchment. Additionally Obi *et al*., (2004) suggested continuous transfer of virulence genes among *E. coli* isolates from human and river sources in rural Venda, South Africa resulting from contamination of river water with human faecal material. These studies suggest that surface water sources such as rivers within South Africa harbour pathogenic *E. coli* and may contaminate irrigated produce thereby posing a food safety and health risk to consumers.

Emergence of previously uncharacterized bacterial foodborne pathogens has also been of major concern worldwide. The *E. coli* 104:H4 outbreak in Germany caused a lot of concern among all stake-holders in food safety (Brzuszkiewicz *et al*., 2011). The strain was identified as a hybrid of EAEC and EHEC possessing the *Stx 2* gene (Brzuszkiewicz *et al*., 2011). *E. coli* has been noted as having a genome which can easily change because of its plasticity (Weiss *et al*., 2011). Therefore there is need to monitor prevalence and characteristics of pathogenic bacteria such as *E. coli* in both irrigation water and on irrigated produce.

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ANTIBIOTIC RESISTANCE IN *E. COLI*

The use of antimicrobials is widespread in many applications such as human and veterinary medicine, animal husbandry, aquaculture, agriculture and food technology (Barbosa and Levy, 2000). The ability of commensal *E. coli* as well as other bacterial organisms to carry resistant genes of clinical importance and their ability to transfer such genes to other bacteria is of greater concern than phenotypic measurements (Lin and Biyela, 2005).

The mechanisms causing acquisition of antibiotic resistance include; alterations of receptor binding sites of drugs, decreased intake of drugs through altering the entry or active flux of a drug, inactivation of the drug and development of resistant metabolic pathways (Levy, 1992). Aquatic environments are reservoirs of a large gene pool of bacterial DNA (Lin and Biyela, 2005). Many viable cells release DNA without cell lysis suggesting that a release of genetic materials may be a normal function of bacteria (Lin and Biyela, 2005). The acquisition of resistance traits may represent a survival mechanism for the microorganisms (Da Silva and Mendonça, 2012). Many antimicrobial genes inserted into conjugative plasmids might be selected by antibiotic selected pressure (Da Silva and Mendonça, 2012). In the aquatic environment, free or bound DNA may be removed by percolation of water, flows of air, water or dust (Lin and Biyela, 2005).

Studies in South Africa have investigated prevalence of antibiotic resistant *E. coli* within surface water sources. In Mmabatho locality, 230 *E. coli* isolates were tested and high antibiotic resistances (>70%) were observed to erythromycin, tetracycline, ampicillin, chloramphenicol and norfloxacin (Kinge *et al*., 2010). Additionally the Mhlathuze river was noted as a major reservoir for antibiotic resistant microbes (Lin and Biyela, 2005). Furthermore Olaniran *et al*., (2009) noted high resistance to antibiotics among *E. coli* isolated from the Palmiet and Umgeni rivers in Durban. The extremely low toxicity of antibiotic classes such as beta-lactams and tetracyclines has led to their indiscriminate use within the medical fraternity leading to the observed high resistance within environmental isolates (Da Silva and Mendonça, 2012). High antibiotic resistance also indicates a negative impact on therapy with classes of antibiotics used (Kinge *et al*., 2010). Therefore the possibility of transmission of resistant genes between bacteria especially those that are pathogenic may pose a health risk to the communities dependent on these river sources (Lin and Biyela, 2005). The transfer of antibiotic resistant pathogens onto irrigated fresh produce may pose increased risk of illness to consumers resulting into severe health complications or fatality.

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RELATIONSHIP BETWEEN ANTIBIOTIC RESISTANCE AND VIRULENCE GENES IN *E. COLI*

According to Da Silva and Medonça, (2012) a lethal pathogen should be virulent, resistant to antibiotics and epidemic. Therefore the continuous use of antibiotics in human/animal medicine and agriculture coupled with improved understanding of the mechanisms of horizontal resistance gene transfer between bacterial species have led to questions regarding association of antimicrobial resistance genes with bacterial virulence determinants (Da Silva and Medonça, 2012). The location of virulence factors and antibiotic resistance genes on mobile genetic structures such as plasmids may facilitate exchange of genetic material between bacterial strains (Da Silva and Medonça, 2012). Therefore in case virulence genetic determinants are located on the same genetic area as antibiotic resistance genes (plasmids, transposons, integrons), they may be co-mobilized under extended exposure to antibiotics (Villa *et al*., 2010).

Resistance to quinolones in *E. coli* has been linked to a loss of virulence factors with an inverse relationship suggested (Da Silva and Medonça, 2012). Additionally emergence of strains producing extended-spectrum beta-lactamases (ESBLs) that cause resistance to Cefotaxime (CXTM-M enzymes) and 3rd generation Cephalosporin Ceftazidime (CTX-M enzymes) have been reported (Da Silva and Medonça, 2012). *E. coli* ST 131 is an example of a clonal group that combines resistance and virulence genes and this property may be linked to its successful dissemination in hospital and community settings worldwide (Johnson *et al*., 2010).

Rasko *et al*., (2011) noted increase in *Stx 2AB* (shigatoxin) by a factor of 80 when the *E. coli* outbreak strain in Germany (C227-11) isolated from an adult human with diarrhoea was subjected to Ciproflaxin (25ng/mm). The link between antibiotic resistance and virulence remains unclear and depends on interactions between the phylogenetic background of the strain and the type of resistant determinant (Da Silva and Medonça, 2012). Unfortunately spread of the successful virulent and resistant strains is occurring worldwide such as the *E. coli* O104:H4 in Germany necessitating more in depth molecular studies on the genetic relationship between antimicrobial resistance and virulence determinants (Da Silva and Medonça, 2012).

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2.3.2 *SALMONELLA* SPECIES

Together with *E. coli* O157:H7, *Salmonella* spp poses equally great concern among health and food safety professionals in ability to cause foodborne illnesses (Olaimat and Holley, 2009). *Salmonella* consists of more than 2400 serotypes (White *et al*., 2002). The 2 recognized species of *Salmonella* are; *Salmonella enterica* and *Salmonella bongori* (Faruque, 2012). *S. enterica* is subdivided into 6 sub species of which 'I enterica' forms the group implicated in more than 99% of all human infections. Salmonellosis in humans is caused by non-typhoidal strains (White *et al*., 2002). Subspecies 'I enterica' consists of subgroups such as; Enteritidis, Typhimurium, Typhi and Chloleraesius (Faruque, 2012).

Salmonella colonizes hosts such as poultry, cattle and pigs later finding its way into food. Its ability to adapt to various environmental conditions through evolutionary processes has now made it possible for adaptation to new environments such as water and fresh produce (Newell *et al*., 2010). For example *Salmonella* spp is able to evolve and fill new niches and respond to environmental changes by undergoing genome degradation where loss of DNA may lead to improved regulation of certain genes, improving several mechanisms and providing new host and environmental habitat opportunities (Law, 2000).

Salmonella is also able to proliferate in soil and on fresh produce for long periods of time even at low temperatures (Matthews, 2009) making it a major food safety threat especially during pre-harvest conditions. The sources of *Salmonella* may include soil, improperly composted manure, handling by workers or irrigation water. The pathogen has been isolated with frequency on tomatoes, seed sprouts and spices (Olaimat and Holley, 2009).

Bacterial attachment to fresh produce may occur through non-hemagglutinating pili, fibrillae and flagella (Patel and Sharma, 2010). *Salmonella* was noted to selectively attach onto cabbage, iceberg lettuce and romaine lettuce (Patel and Sharma, 2010). Additionally *Salmonella* was isolated from fresh produce irrigated with water from the Loskop canal (Ijabadeniyi *et al*., 2011b).

2.3.3 *LISTERIA* SPECIES

The Genus *Listeria* is composed of Gram positive, oxidase negative and catalase positive non-sporulating rods (Ells and Hansen, 2006). It is widely distributed in the environment with presence in soils, decaying vegetation, animal faeces, sewage, silage, water and intestinal tracts of humans and animals (White *et al*., 2002). *L. monocytogenes* is the only species associated with disease in humans (Ells and Hansen, 2006) causing listeriosis which can be

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fatal especially among the young, old and immune-compromised (Faruque, 2012). Outbreaks of listeriosis have been linked to refrigerated ready-to-eat foods that are consumed without heating (White *et al*., 2002). Fresh produce such as lettuce may form part of this category especially if consumed as a salad or sandwich.

High prevalence of *L. monocytogenes* was noted in irrigation water from the Loskop canal, Olifants and Wilge rivers (Ijabadeniyi *et al*., 2011b). Additionally high prevalence of the pathogen was noted in irrigation water from two Western Cape rivers (Lötter, 2010). However, low prevalence of *L. monocytogenes* was noted in fresh ready to eat vegetables collected from a market in Spain (Badosa *et al*., 2008). An outbreak of *L. monocytogenes* associated with cantaloupes occurred in the Colorado State, United States (US) leading to recall of a large amounts of produce from the region (CDC, 2011).

2.3.4 *BACILLUS CEREUS (B. CEREUS)*

B. cereus is a food pathogen known to cause disease through production of enterotoxins (Elhariry, 2011). The enterotoxins cause food related illnesses when levels of *B. cereus* multiply to greater than 10^6cfu/g (Elhariry, 2011). Toxins produced by the pathogen also cause gastrointestinal illness (Newell *et al*., 2010). *B. cereus* produces spores which under favourable conditions germinate and convert to actively growing vegetative cells that can cause disease (Elhariry, 2011). The spores of *Bacillus* spp are heat resistant and can survive mild heat treatments (Newell *et al*., 2010) making them a potential risk for causing disease when present on fresh produce such as lettuce which usually does not undergo any postharvest heat treatment. Many infections caused by *B. cereus* go unnoticed because of lack of diagnostic tools (Newell *et al*., 2010).

2.4 FOODBORNE OUTBREAKS ASSOCIATED WITH FRESH PRODUCE

Fresh produce has been associated with more food related outbreaks than any other food source over the last 10 years (Warriner and Namvar, 2010). Common examples of fresh produce associated with these outbreaks include among others; lettuce, sprouts, melons, tomatoes and herbs (Sapers and Doyle, 2009). For example in the US, increase in fresh produce related foodborne outbreaks has been linked to increased consumption of uncooked vegetables in salads, sandwiches, ready-to-eat food bars and ethnic foods (Seiber, 2012).

Additionally, increase in fresh cut and minimal processing of fruits and vegetables could also lead to this increase (Seiber, 2012). According to Matthews (2009), lettuce has been linked to the majority of outbreaks caused by fresh produce in the United States.

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Lettuce is highly susceptible to contamination with foodborne pathogens probably because the edible part (leaf) grows in close proximity to the soil. Additionally lettuce is eaten raw most of the time. Figure 1 shows how consumption of fresh fruits and vegetables may lead to foodborne diseases. The relationships are described as follows: isolation of outbreak pathogens from produce and irrigation water (links B and A), as well as the actual source (links C, B, and A); epidemiological investigations of food poisoning outbreaks implicating irrigated produce (links B and A), or contamination of produce via other vectors (links D and A); observations of increased incidence of disease in areas practicing irrigation utilizing highly contaminated wastes (link E).

In the two year duration of this study (2011 to 2012), some major foodborne outbreaks occurred around the world. An outbreak of enteroaggregative shigatoxin producing *E. coli* O104:H4 occurred in Germany and across Europe affecting more than 3000 people who showed symptoms of gastroenteritis and haemolytic uremic syndrome with notable fatalities (Rohde *et al*., 2011). Emergence of this lethal strain of *E. coli* was suggested to have been linked to horizontal genetic exchange due to plasticity of the *E. coli* bacterial genome (Rasko *et al*., 2011). Additionally an outbreak linked to *L. monocytogenes* occurred in different states within the United States and its source was traced back to cantaloupes (CDC, 2011). The outbreak due to *L. monocytogenes* caused illness in 147 individuals (CDC, 2011). In 2012, 29 individuals across 11 states yet again in the United States were affected by an outbreak of *E. coli* O26 with a number of them having eaten spouts at a local restaurant (CDC, 2012a).

In South Africa, The Center for Enteric Diseases at the National Health Laboratory Service monitors diseases caused by *Salmonella* spp and EHEC (National Institute for Communicable Diseases (NICD), 2012).

On collecting provisional data over a 2 year period beginning 2011, results show low prevalence of *Salmonella* spp and EHEC (NICD, 2012). However since data was collected from different sources, the proportion attributed to food is unknown.

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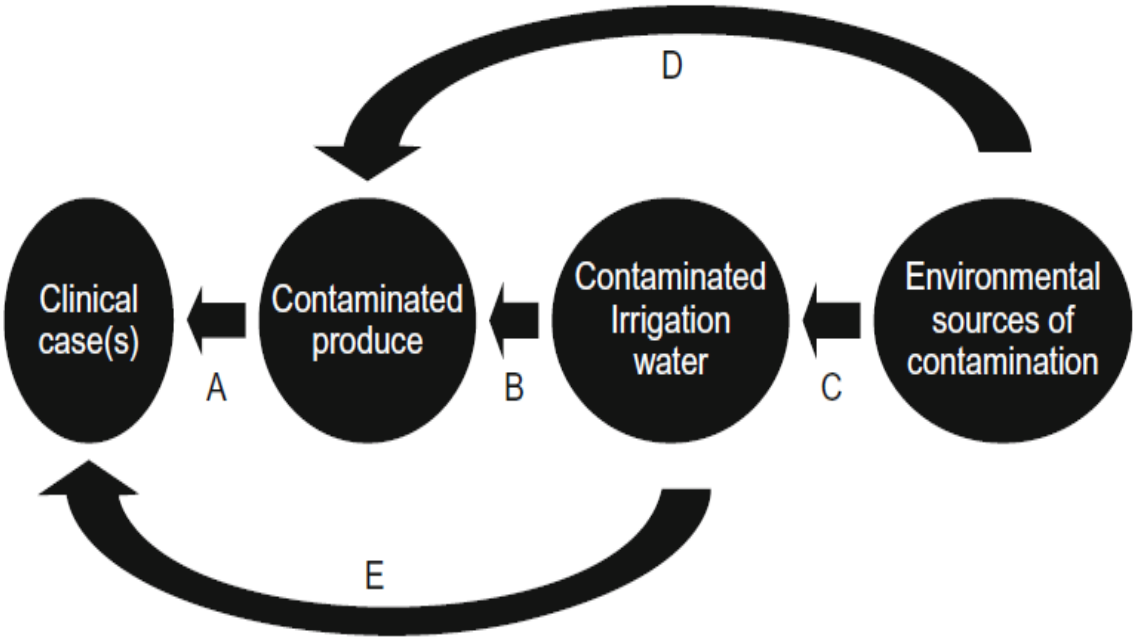

Figure 1: Inferences in research of irrigation water as a source of foodborne diseases caused by consumption of fruits and vegetables (Pachepsky *et al*., 2011)

Data relating to foodborne outbreaks is limited to a few industrialized countries which also limit their surveillance to a few pathogens yet proper surveillance requires continuous monitoring of outbreaks and pathogen prevalence over a long period of time going back more than 20 years (Newell *et al*., 2010). For example on comparing peer reviewed research by different scholars around the world relating to food safety in fresh produce, (Ilic *et al*., 2012), reported that 65% of it carried out in the US, Europe or Canada. This left a number of unanswered questions relating to safety of fresh produce sourced from other countries especially those categorized as developing and least developed.

Foodborne outbreaks may have a number of other negative effects among such as; damage to patients including lost income, long term damage to a company's reputation and damage to an entire segment of the produce industry such as the Salinas Valley in California, USA (Sapers and Doyle, 2009) and the Western Province in South Africa (Ackermann, 2010). For example during the *E. coli* O104:H4 outbreak in Europe, 812 million Euros were lost by farmers in the fruit and vegetable industry within the first two weeks of the outbreak (Commission of the European Communities, 2011).

To determine the source of a foodborne outbreak, investigators need to genetically match laboratory confirmed pathogens from ill individuals with pathogens on foods while

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co-currently determining where and how contamination occurred (Sapers and Doyle, 2009). However foodborne outbreak investigations are usually hindered by delays and variability in factors such as; diagnostic testing procedures, reporting of results, conducting of epidemiological investigations, perishable goods and poor record keeping (Sapers and Doyle, 2009). Such factors may be responsible for the inadequate data collected on prevalence and monitoring of foodborne illnesses and outbreaks in many countries especially those which are resource strained in both financial and human capita (Ilic *et al*., 2012).

2.5 SOURCES OF FOODBORNE BACTERIAL PATHOGENS

There are a number of environmental factors that influence the microbial quality of irrigation water. These include; agriculture, wildlife and human inputs (Pachepsky *et al*., 2011). Figure 2 shows some of the mechanisms through which irrigation water maybe contaminated. Animals can contaminate the water and under favourable conditions bacterial numbers may increase placing irrigated crops under risk of contamination (Matthews, 2009). In relation to fresh produce, a number of factors can lead to contamination such as; environments associated with animal production, faeces from wild animals, composted manure, soil, run off and irrigation water (Sapers and Doyle, 2009).

Salmonella spp and EHEC are two pathogens most commonly implicated in foodborne outbreaks around the world. Ruminant manure and sewage have been named as major sources of *Salmonella* spp and *E. coli* O157:H7 (Olaimat and Holley, 2009).Cattle infected with *E. coli* O157:H7 has been noted to excrete up to 10^7 cfu/g of faecal material (Solomon and Sharma, 2010). Such high numbers are regularly excreted by cattle referred to as ‘super shedders’ (Solomon and Sharma, 2009). Incidence of *E. coli* O157:H7 in cattle is reportedly increased by feeding the animals on a high energy diet containing starch (Weiss *et al*., 2011). Other sources of Shigatoxin producing *E. coli* include; pigs, dogs, chicken and turkeys with environmental persistence of the pathogen being fuelled by constant shedding coupled with infection and re-infection of different animal sources (Berry and Wells, 2010).

Du Preez *et al*., (2008) reported on the transfer of related and unrelated *E. coli* isolates between storage water points and river water within a rural South African community suggesting persistence of the microorganism among closely linked points. On the other hand high incidence of *Salmonella* spp occurs when water sources serving large populations become contaminated with sewage (Faruque, 2012). Studies reporting on potential sources of bacterial food borne pathogens depict potential for these disease causing agents to withstand

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environmental conditions thus finding ways to enter the food chain posing a risk to food safety and public health.

In a bid to limit influx of bacterial foodborne pathogens, surface and ground water sources should be protected from potential contamination sources including wildlife, animal waste, run off, human activity and sewage or industrial effluent (Olaimat and Holley, 2009). Proper water treatment is able to limit spread and transmission of EHEC (Weiss and Schmidt, 2011). The period after irrigation prior to harvest has been noted as crucial in limiting persistence of bacterial pathogens on irrigated produce. For example the US Environmental Protection Agency (EPA) requires continuous monitoring of water used for irrigation especially if it has not been treated (EPA, 2005) such as that from river sources.

Whether the aforementioned sources of contamination present an actual food safety risk depends on the extent to which human pathogens in the farm environment interact with fresh produce surfaces, adhere to them, survive environmental stresses in the field and subsequent exposure to sanitizing processes (Sapers and Doyle, 2009). The robustness of these pathogens may favour population growth to a level sufficient to cause disease.

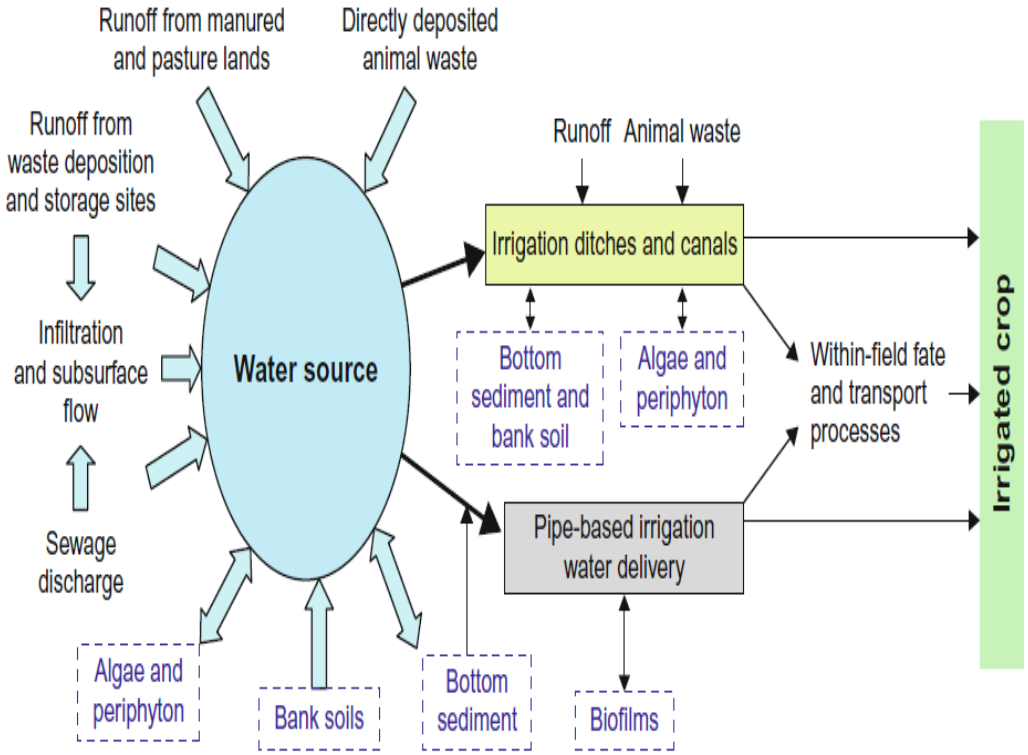

Figure 2: The layout of the processes affecting the quality of irrigation waters (Pachepsky *et al*., 2011)

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2.6 ENTERIC PATHOGENS AND THE PLANT PHYLLOSPHERE

Enteric pathogens are transmitted through the faecal-oral route (Al-Sa´ed, 2007) and therefore are transient micro-organisms in the phyllosphere which forms part of the aerial structures of plants (Aruscavage, 2007). Some studies have investigated the incidence and prevalence of microorganisms inhabiting the phyllosphere of fresh produce. Ibekwe and Grieve, (2004) isolated *Pseudomonas* spp, *Acidobacterium* spp, *Bacillus* spp and *Agrobacterium* spp from the phyllosphere of fresh produce. Similarly Lötter (2010) isolated a number of bacterial pathogens such as *L. monocytogenes*, *S. aureus*, *Enterobacter aerogenes*, *E. cloacae*, *Klebsiella pneumonia* and *E. coli* from fresh produce. There are a number of factors that influence proliferation of these microorganisms on fresh produce. They include; product type, cultivar, physiological state of plant and type of pathogen (Critzer and Doyle, 2010).

The topography of the phyllosphere influences proliferation by facilitating attachment of pathogens. For example during a study using different strains of *L. monocytogenes*, (Ells and Hansen, 2006) noted that the pathogen attached preferentially to the leaf surfaces predominantly at folds and crevices. The type of plant cultivar may also influence pathogen attachment by providing points of contact (Arucscavage, 2007). Low attachment of *Salmonella* spp to tomatoes and lettuce was noted indicating that the pathogen may not readily attach to the phyllosphere (Critzer and Doyle, 2010). However higher counts of *E. coli* were noted on beans and peas compared to tomatoes which was attributed to the smooth surface of the tomato epidermis that made attachment difficult (Ackermann, 2010).

Infection with biotropic plant pathogens may alter the physiological state of plants by causing lesions which change abundance and composition of exudates on plant surfaces (Aruscavage, 2007). In a study to determine the interaction of plant pathogens with *E. coli* O157, (Aruscavage, 2007) noted that plant pathogens increased nutrient availability and provided attachment sites for the pathogen. Additionally *Salmonella* is attracted and able to metabolize nutrients contained within the apoplastic fluids of plants (Warriner and Namvar, 2010). New habitats to which pathogens are increasingly exposed may provide opportunities for evolution (Newell *et al*., 2010). Salmonellosis today is more associated with fresh produce around the world (Newell *et al*., 2010) than animal products such as beef and poultry. Attachment of *Salmonella* to fresh produce is through virulence gene mechanisms such as the type III secretion system (Newell *et al*., 2010).

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Similar to *E. coli* O157:H7, *Salmonella* has epitopes used for binding to plant structures such as stomata (Warriner and Namvar, 2010).

The mechanisms by which bacteria attach to plant surfaces are highly complex (Ells and Hansen, 2006). Three mechanisms have been put forward; biofilm formation, use of flagella and interaction with other bacteria (Critzer and Doyle, 2010). Figure 3 shows some of the different mechanisms through which enteric foodborne pathogens may attach to fresh produce. *Bacillus* spp, *Listeria* spp, *Staphylococcus* spp and *Escherichia* spp are capable of forming biofilms (Elhariry, 2011). Adherence factors such as flagella (Welch, 2006) may help in attachment to plant surfaces. The interaction/competition between natural flora and enteric pathogens has also been noted on lettuce (Moyne *et al*., 2011).

Aeromonas spp and *Pseudomonas* spp together with Gram negative bacteria were noted to have inhibitory effects on enteric bacterial pathogens such as *E. coli* O157:H7 by producing acid and antimicrobial peptides (Critzer and Doyle, 2010).

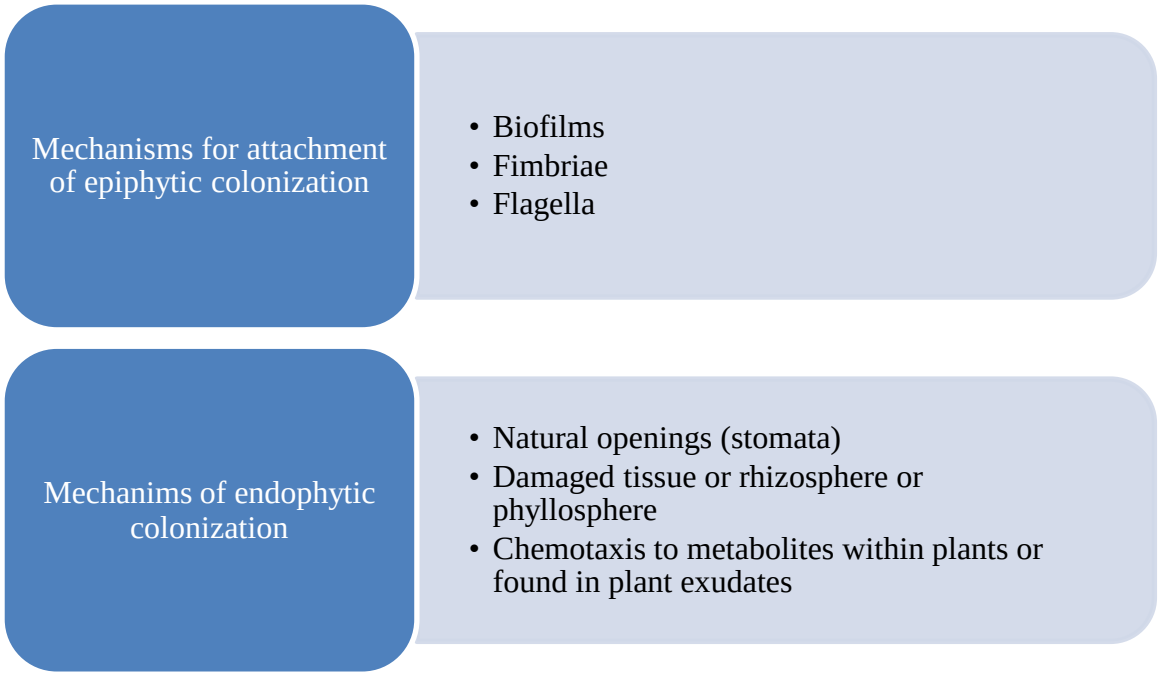

Figure 3: Colonization of fresh fruits and vegetables by enteric foodborne pathogens (Critzer and Doyle, 2010)

Studies such as those previously noted have shown that enteric bacterial pathogens may proliferate in the phyllosphere. Post-harvest sanitation mechanisms such as washing with chlorine cannot remove all bacterial pathogens from fresh produce (Johnston *et al*., 2006; Solomon and Sharma, 2009).

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B. cereus spores can survive harsh conditions such as washing with chlorine, germinating when favourable conditions are presented (Elhariry, 2011).Therefore limiting pre-harvest bacterial contamination of fresh produce that undergoes minimal processing may reduce the risk of disease to the final consumer.

2.7 MICROBIAL SOURCE TRACKING (MST)

Determining the correct source of faecal contamination is important in order to maintain quality of surface water bodies. Faecal indictor bacteria such as faecal coliforms, *E. coli* and faecal streptococci have been used to indicate faecal contamination in food and the environment. What is important for public health is distinguishing faecal bacteria from human and animal sources (Vantakaris *et al*., 2006).

Using different methods for identifying sources of faecal indicator bacteria is termed as MST (Whitlock *et al*., 2002). However the complexity of environmental conditions has hindered the application of a single method to all settings (Jan and Sadowsky, 2007). MST is a wide field and keeps changing continuously as newer methods are found to improve on reliability of results (EPA, 2005).

Microbial source tracking methods that have been applied to water sheds include phenotypic and genotypic methods (Burnes, 2003; Vantakaris *et al*., 2006; Mohapatra and Mazumder, 2008; Da-Silva *et al*., 2011). Among the phenotypic methods used for microbial source tracking is antibiotic resistance of faecal coliforms isolated from water sources (Burnes, 2003). The use of antibiotics is either based on Multiple Antibiotic Resistance (MAR) index or Antibiotic Resistance Analysis (ARA) (Olivas and Faulkner, 2008). MAR involves testing *E. coli* or faecal coliform resistance to multiple antibiotics at a single concentration while ARA profiles are developed by testing the same faecal indicators to multiple antibiotics at increasing concentrations (Olivas and Faulkner, 2008).

Use of antibiotics is based on measurable differences in resistance among bacteria isolated from faeces of humans and animals allowing for discrimination (Whitlock *et al*., 2002). Da-Silva *et al*., (2011) used MAR to determine the anthropogenic influence at different points along a river in Brazil. Higher resistance to antibiotics was noted in *E. coli* recovered from dense than sparsely populated areas (Da Silva *et al*., 2011). Additionally *E. coli* from humans have higher resistance to antibiotics than animal isolates (Vantakaris *et al*., 2006). Use of antibiotic resistance has provided good indication of possible sources of faecal contamination in water sources (Burnes, 2003; Vantakaris *et al*., 2006). The popularity of this

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method in source tacking studies is ease to carry out negating need for expensive equipment (Vantakaris *et al*., 2006). Drawbacks linked to use of antibiotics is that application in big water sheds might not give a good indication of faecal sources due to within source variation and therefore better results are obtained if complimented with a finger printing method (Jan and Sadowsky, 2007). The use of a wider variety of antibiotics in a study has also been noted to improve discrimination among isolates (Vantakaris *et al*., 2006).

Genotypic methods used for source tracking in water sheds include; Repetitive Extragenic Palindromic (REP)-PCR, Amplified Fragment Length Polymorphism (AFLP) and Pulsed-Field Gel Electrophoresis (PFGE) (Seurinck *et al*., 2005).

These methods have successfully been used in water sheds to help show genetic relatedness among faecal indicator bacteria and the source of contamination (Seurinck *et al*., 2005). Repetitive extragenic palindromic-Polymerase Chain Reaction (Rep-PCR) is a genotypic fingerprinting method that generates specific strain patterns by amplifying repetitive elements in the *E. coli* genome (Mohapatra and Mazumder, 2008). Rep-PCR involves use of repetitive elements such as repetitive extragenic palindromic (REP) sequences, enterobacterial repetitive intergenic consensus sequences (ERIC) and Box sequences which have been reported as highly evolutionary conserved as they are sites for essential protein-DNA interaction (Suerinck *et al*. 2005). Mohapatra and Mazumder (2008) compared the efficiency of 5 different Rep-PCR methods to differentiate faecal *E. coli* strains according to their source of origin (human or animal).

AFLP is a genotypic fingerprinting method that uses a combination of genomic DNA digestion with resolution enzymes and PCR with short adaptors fixed to the digested fragment end providing sufficient length of known sequence for primers that are to be used for PCR (EPA, 2005). AFLP was used to determine the genetic relatedness of *E. coli* isolates recovered from closely related points within a domestic setting in rural South Africa (Du Preez *et al*., 2008). Possible routes for contamination within a rural household setting were determined (Du Preez *et al*., 2008).

PFGE involves pulse field gel electrophoresis of total genomic DNA after restriction enzyme digestion using infrequently cutting enzymes (EPA, 2005). PFGE was used to show genetic relatedness among *E. coli* from a water source and associated sediment (Lu *et al*., 2004). PFGE was found to have high resolution though this made it harder to ably discriminate

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between isolates hence the study recommended using a complimentary method especially with studies involving large water sheds (Lu *et al*., 2004).

The major drawbacks of using genotypic methods is the intensive laboratory work involved and expensive equipment which makes them out of reach for most laboratories (Field *et al*., 2003; Seurinck *et al*., 2005). Suggestions have been made that future microbial source tracking methods should be easy to carry out and combine the advantages of both phenotypic and genotypic characters (Scott *et al*., 2002).

Therefore a study that combines use of these two (phenotypic and genotypic) inherent characteristics of faecal indicator bacteria would provide higher resolution for analysis and more reliable information relating to sources of faecal contamination.

2.8 CONCLUSION

Surface water sources such as rivers provide irrigation water which is used for growing fresh produce however their deteriorating bacterial quality poses a risk of contaminating irrigated produce. Irrigated fresh produce contaminated with enteric bacterial pathogens may cause illness if postharvest processing fails to eliminate all pathogens prior to consumption. Pathogenic factors in bacteria such as antibiotic resistance and virulence have been noted as major risks to food safety and public health as witnessed by the increased foodborne outbreaks worldwide. The versatility and lethality of these new emerging pathogens previously not associated with fresh produce necessitates the need for further investigation into factors favouring acquisition of such characters within bacteria.

Through continuous microbiological monitoring of irrigation water quality, trends can be observed and inferences made on possible sources of contamination and mitigation measures proposed. With the increasing trade in fresh produce around the world possible contamination points such as irrigation water need to be assessed in order to limit and control foodborne outbreaks. South Africa as a leading economy is Africa and a new member of the (Brazil, Russia, India and China) BRICs economic block is strategically positioned to benefit from new markets for its fresh produce of which it is a major producer and exporter. Therefore investigating measures that may provide information on production of safe fresh produce such as efficient use of good quality irrigation water will go far in strengthening capacity within the fresh produce industry.

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2.9 OBJECTIVES AND HYPOTHESES

2.9.1 HYPOTHESES

1. Irrigation water from the Skeerpoort river (North West province) flowing through an urban area will have higher prevalence of bacterial pathogens than irrigation water in Loskop dam canal (Mpumalanga province) which flows through a predominantly rural area. In South Africa, rivers flowing through urban areas are at high risk of pathogenic bacterial contamination from failing sewage systems that often lack adequate waste disposal facilities within many communities' especially informal settlements, thereby making rivers disposal sites for domestic and industrial effluent (Britz *et al*., 2012; Olaniran *et al*., 2009). Additionally untreated sewage and run-off contains large amounts of organic and inorganic nutrients which may favour proliferation of pathogenic bacteria such as *E. coli* and *Salmonella* spp (Korajkic *et al*., 2010).
2. Contaminated irrigation water from the Skeerpoort river will transfer bacterial foodborne pathogens onto irrigated lettuce. Bacterial foodborne pathogens such as *E. coli* and *Salmonella* spp may use attachment structures called flagella (Welch, 2006) and virulence factors such as the type III secretions system (Newell *et al*., 2010) to attach onto fresh produce. Additionally *E. coli* and *Bacillus* spp are capable of forming biofilms with indigenous bacteria of the plant which may facilitate pathogen proliferation (Critzer and Doyle, 2010) on fresh produce. Irrigation water from the Loskop canal (Ijabadeniyi *et al*., 2011b) and the Skeerpoort river (Duhain, 2011) has previously been reported as a source of bacterial foodborne pathogens on irrigated vegetables.
3. Irrigation water from the Loskop canal and the Skeerpoort river will harbour *E. coli* possessing antibiotic resistant and virulence genes. Additionally irrigation water from the Skeerpoort river containing *E. coli* with antibiotic and virulence genes will be a source of contamination on irrigated lettuce. Water environments are reservoirs of a large gene pool of bacterial DNA (Lin and Biyela, 2005) therefore widely used antibiotics within sectors such as agriculture (Barbosa and Levy, 2000) could end up in irrigation water sources and subsequently on irrigated fresh produce. The acquisition of antibiotic resistant and virulence genes by insertion on conjugative plasmids may represent a survival mechanism *by E. coli* to environmental conditions such as water percolation (Da Silva and Medonca, 2012). Virulent genetic determinants located on the same genetic areas as those for antibiotic resistance

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(plasmids, transposons, integrons) maybe co-mobilized under extended exposure to antibiotics (Villa *et al*., 2010). *E. coli* has attachment structures such as flagella which may facilitate colonisation and finally survival on secondary environments such as the phyllosphere of fresh produce (Aruscavage *et al*., 2008; Ampadu, 2007).

2.9.2 OBJECTIVES

1. To determine the prevalence of bacterial foodborne pathogens in irrigation water collected from the Skeerpoort river and Loskop dam canal based on proximity to an urban and predominantly rural area respectively.

2. To determine whether irrigation water from the Skeerpoort river is a source of bacterial foodborne pathogens on irrigated lettuce grown under environmental field conditions.

3. To determine whether *E. coli* isolated from irrigation water in the Loskop canal and the Skeerpoort river harbours antibiotic resistant and virulence genes. Additionally to determine whether irrigation water from the Skeerpoort river is a source of contamination on irrigated lettuce with *E. coli possessing* antibiotic resistant and virulence genes.

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CHAPTER 3: RESEARCH

3.1 INTRODUCTION

This study was divided into 2 phases that aimed at assessing the bacteriological quality of irrigation water from two South African irrigation water sources, the Loskop canal (the Mpumalanga province) and the Skeerpoort river (North West province). Additionally irrigation water from the Skeerpoort river was investigated as a source of bacterial pathogens on lettuce grown under environmental field conditions. Phase 1 involved determining the level of bacterial indicators, incidence of foodborne pathogens as well as aerobic bacterial composition of irrigation water from the two irrigation water sources and on irrigated lettuce over 10 months (January to October, 2011) (Figure 4). Phase 2 involved determining antibiotic resistance and virulence genes in *E. coli* isolated from the two irrigation water sources and on irrigated lettuce (Figure 5).

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EXPERIMENTAL DESIGN

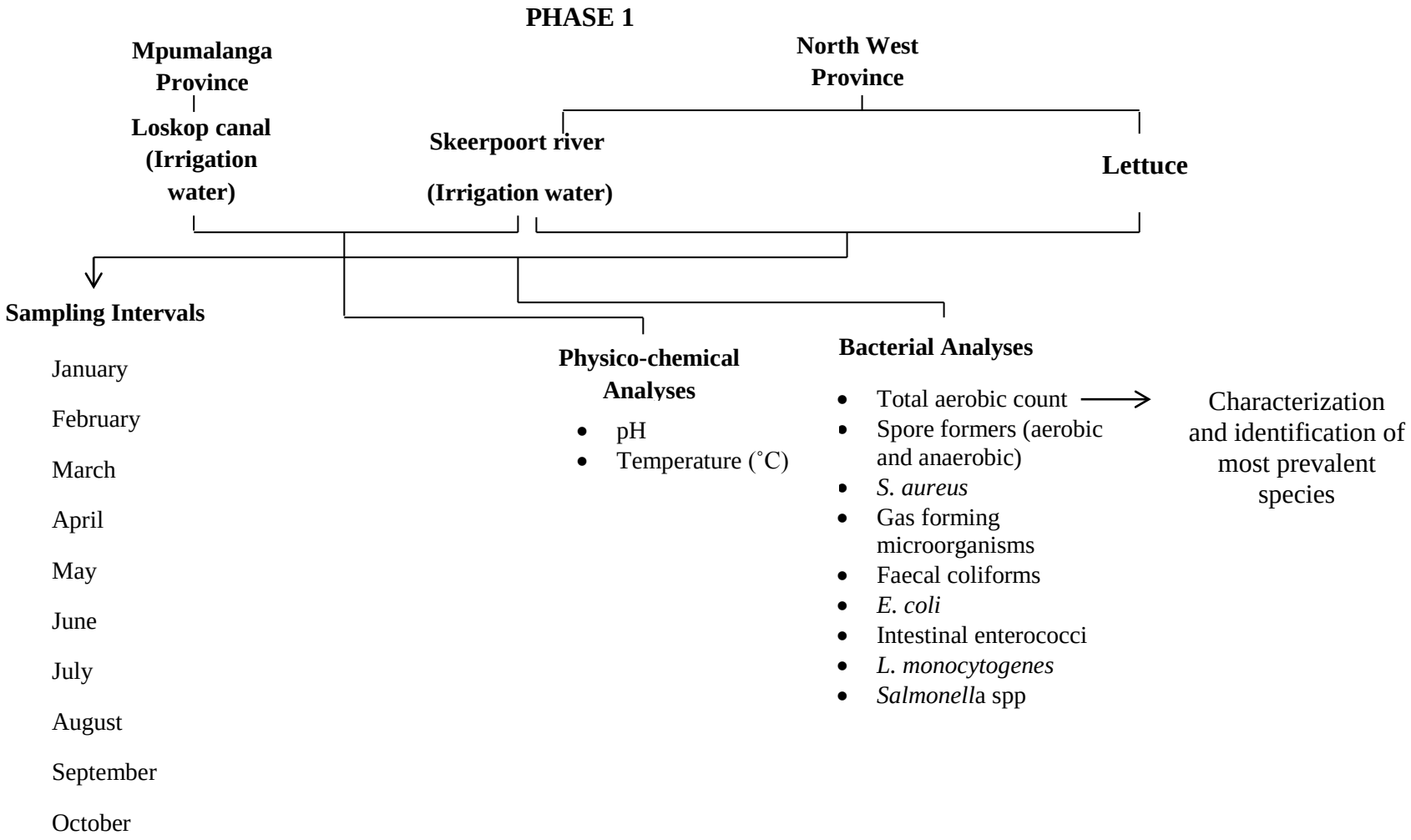

Figure 4: Experimental design showing bacterial analyses of irrigation water from the Loskop canal, the Skeerpoort and lettuce irrigated with water from the Skeerpoort river over a 10 month sampling period

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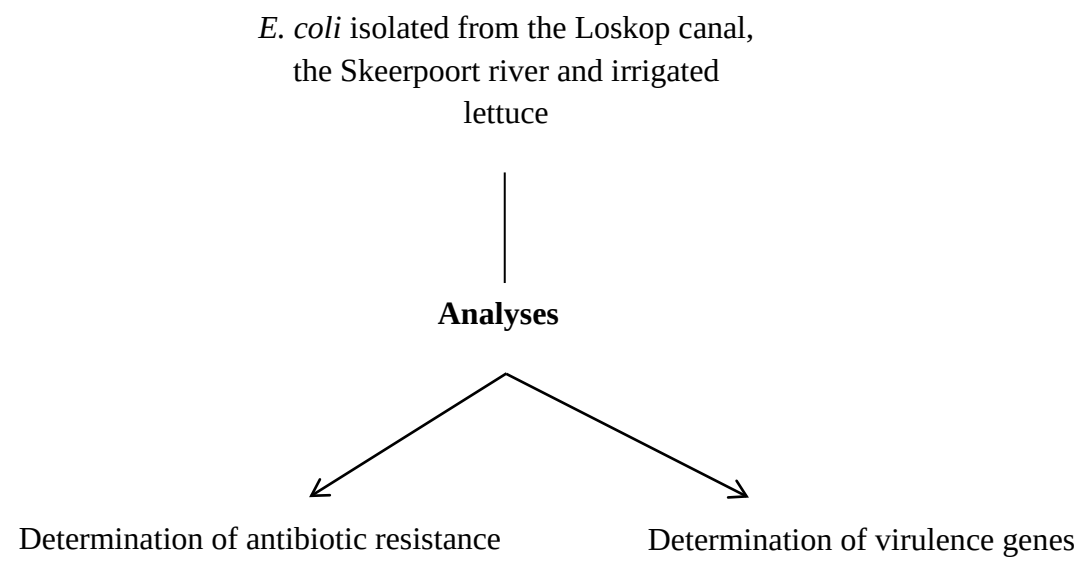

Figure 5: Experimental design showing analyses for characterization of *E. coli* isolated from the Loskop canal, the Skeerpoort river and lettuce irrigated with water from the Skeerpoort river

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3.2 BACTERIAL COMPOSITION OF IRRIGATION WATER FROM THE LOSKOP CANAL, SKEERPOORT RIVER AND IRRIGATED LETTUCE

ABSTRACT

The bacteriological quality of two irrigation water sources in South Africa (Loskop canal and Skeerpoort river) as well as lettuce irrigated with water from the Skeerpoort river was determined monthly over 10 months. Counts for Aerobic colony counts (ACC), Aerobic spore formers (ASF), Anaerobic spore formers (AnSF), Faecal coliforms (FC), Intestinal enterococci (IE) and *Staphylococcus aureus* (*S. aureus*) as well as prevalence of *Escherichia coli (E. coli*), *Salmonella* spp and *Listeria monocytogenes* (*L. monocytogenes*) were determined. Additionally the most prevalent aerobic bacterial species isolated from the three sources were determined. Higher mean rainfall was noted in areas surrounding the Skeerpoort river (74.7mm) than the Loskop canal (0.1mm). Mean water temperature was 15.4˚C and 18.2˚C while mean pH was 7.4 and 8.4 in the Loskop canal and the Skeerpoort river respectively. Low mean bacterial counts <3.4 $\log_{10}$cfu/ml were noted for ACC, ASF, AnSF, *S. aureus* and IE at both irrigation sites. Higher mean ACC were noted on lettuce (5.9 $\log_{10}$cfu/g) than in irrigation water from the Skeerpoort river (3.4 $\log_{10}$cfu/ml). Similarly higher *S. aureus* counts were noted on lettuce (3.0 $\log_{10}$cfu/g) than irrigation water from the Skeerpoort river (0.5 $\log_{10}$cfu/ml). Although low mean counts of FC (1.3 $\log_{10}$cfu/100ml) were noted for all three sources, high incidence of *E. coli* was observed during bacterial composition studies on non-selective media. This suggested underestimation of faecal contamination possibly indicating that identification of specific pathogens provided a better measure of assessing bacterial contamination than bacterial indicators.

E. coli, *Bacillus* spp and *Enterobacter* spp were the most prevalent genera in the Loskop canal, the Skeerpoort river and on lettuce. Prevalence of *E. coli*, *Bacillus* spp and *Enterobacter* spp in the Loskop canal was 23%, 33% and 26% respectively. Prevalence in the Skeerpoort river was 36%, 26%, 16% respectively. On lettuce prevalence was 36%, 30% and 6.7% respectively. *E. coli* O157:H7 was isolated at both irrigation sites while *Salmonella enterica* (gp 1) ST *paratyphi* A was isolated from the Skeerpoort river. The isolation of similar bacterial pathogens in irrigation water from the Skeerpoort river and on irrigated lettuce suggested possible contamination of the produce by water. Additionally it suggests ability of bacterial pathogens to withstand environmental conditions which may pose a risk to food safety and public health among individuals consuming irrigated fresh produce.

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3.2.1 INTRODUCTION

Most South African water requirements are provided by surface water supplies with irrigation as the major user (DWA, 2012). According to the WRC (2012), over 80% of South African river eco-systems are threatened. Faecal pollution lowers the bacteriological quality of surface water sources because it usually contains enteric bacterial pathogens such as *E. coli* and *Salmonella* spp (Korajkic *et al*., 2010). Studies on South African rivers have shown that they pose a risk of transferring enteric bacterial pathogens onto irrigated produce (Tshivhandekano, 2006; Olaniran *et al*., 2009; Ijabadeniyi *et al*., 2011b; Duhain, 2011). Irrigated fresh produce undergoes minimal postharvest processing which does not remove all bacterial contamination (Pachepsky *et al*., 2011). Therefore the risk of illness especially if contaminated with *E. coli* O157:H7, which has a low infective dose, <100 viable cells may increase (Doyle and Erickson, 2008).

In 2011 two major foodborne outbreaks associated with fresh produce occurred in the United States and Europe (CDC, 2011). Such outbreaks increase government and public concern regarding the safety of fresh produce thereby prompting the need to enact measures that can limit pathogenic bacterial contamination, especially under field conditions. Foodborne pathogens have been shown to attach to fresh produce thorough a number of mechanisms (Aruscavage *et al*., 2008; Ijabadeniyi *et al*., 2011a). Therefore these pathogens have the ability to proliferate in a secondary environment such as the plant phyllosphere and could survive minimal processing such as washing with chlorine thereby remaining viable on packaged ready to eat fresh produce. Such produce may present a risk of causing illness to consumers (Pachepsky *et al*., 2011).

The location of a water source can influence its microbiological quality (Korajkic *et al*. 2010). This is probably because different land-use patterns can affect pathogen concentration in water sources (Pachepsky *et al*., 2011). Higher pathogenic bacterial contamination has been noted at points along rivers closer to human settlements as opposed to those in close proximity to sparsely populated areas (Gerba and Choi, 2009; CSIR, 2010; Da-Silva *et al*., 2011; Walters *et al*., 2011).

The supply of water to communities in South Africa has not been matched by provision of sewage infrastructure for proper disposal leading many communities' especially informal settlements to use rivers as disposal points (Britz *et al*., 2012).

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Studies have reported on the deteriorating bacteriological quality of rivers flowing through urban areas in South Africa (Olaniran *et al*., 2009; Gemmel and Schmidt, 2012). Previous studies have also reported on high bacterial contamination in water sources located in rural areas (Obi *et al*., 2002; Obi *et al*., 2004; Du Preez *et al*., 2008; Ijabadeniyi *et al*., 2011b). Therefore this study aimed to determine the bacteriological quality and composition of two South African irrigation water sources, the Loskop canal and the Skeerpoort river located in different provinces and therefore possibly exposed to different land use practices and sources of bacterial contamination. Additionally, the study investigated irrigation water from the Skeerpoort river as a source of bacterial contamination on irrigated lettuce grown under field conditions.

3.2.2 MATERIALS AND METHODS

SAMPLING SITES

Irrigation water was collected from the Skeerpoort river (North West Province) and the Loskop canal (Mpumalanga Province). The Skeerpoort river provides irrigation water for growing fresh produce and the sampling site was downstream the Hartbeespoort dam, a major tourist location with an urban settlement. The Loskop canal carries irrigation water throughout Loskop irrigation scheme which is a predominantly rural area. The canal is fed with water from the Olifants and Wilge rivers (Ijabadeniyi *et al*., 2011b).

WATER SAMPLING

One water sample was collected monthly from the Loskop canal and the Skeerpoort river over 10 months from January to October, 2011. Water samples were collected aseptically in sterile plastic bottles for physico-chemical and bacteriological analyses and transported to the Department of Food Science, University of Pretoria and analysed within 6 hours.

LETTUCE SAMPLING

A lettuce sample was collected monthly from a field irrigated with water from the Skeerpoort for 10 months from January to October, 2011. Samples were transported to the Department of Food Science, University of Pretoria and analysed upon arrival.

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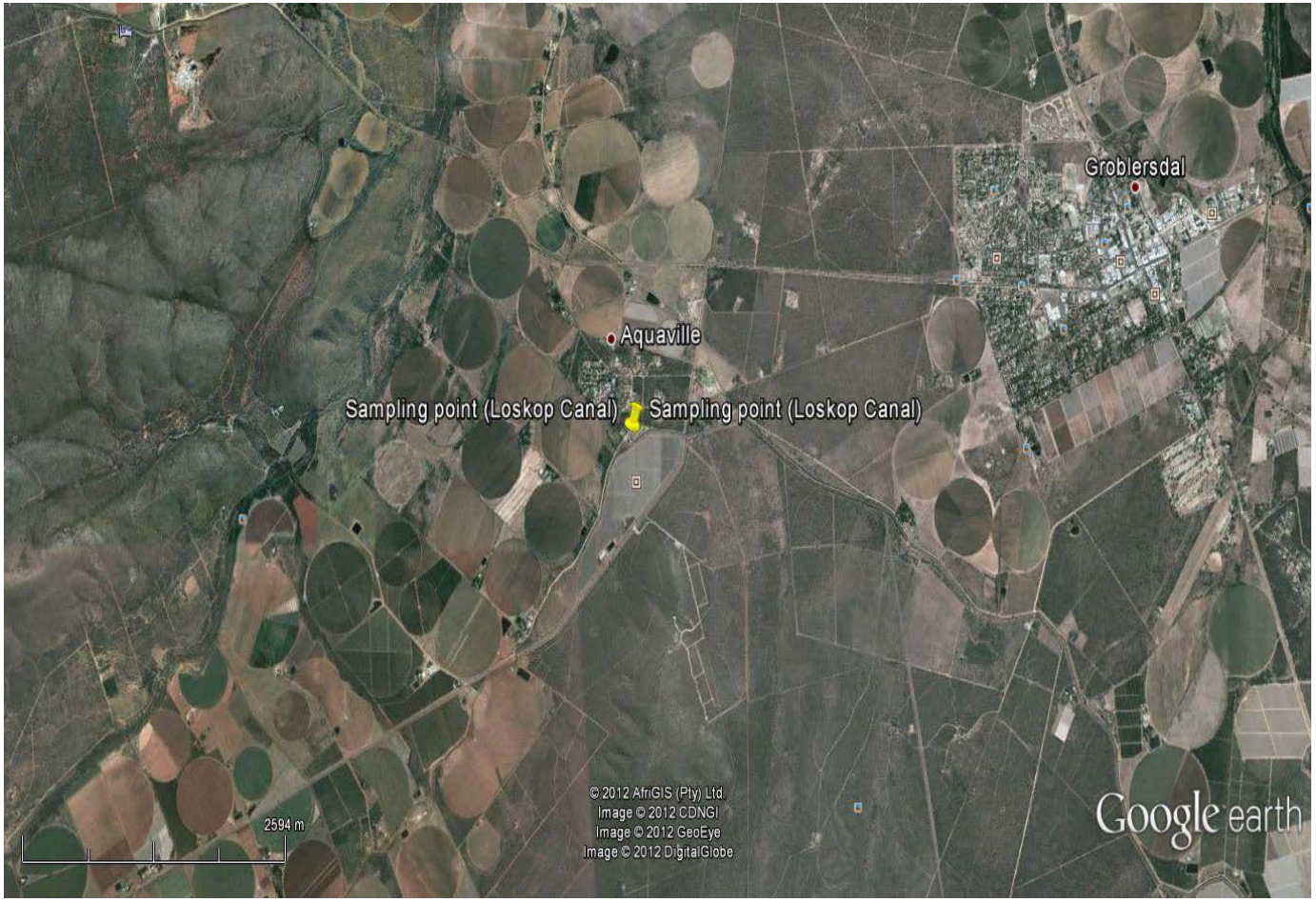

Figure 6: Sampling location along the Loskop canal (Source: Google Earth). Accessed: 20/12/2012. The Loskop canal carries irrigation water throughout Loskop irrigation scheme which is a predominantly rural area. The canal is fed with water from the Olifants and Wilge rivers

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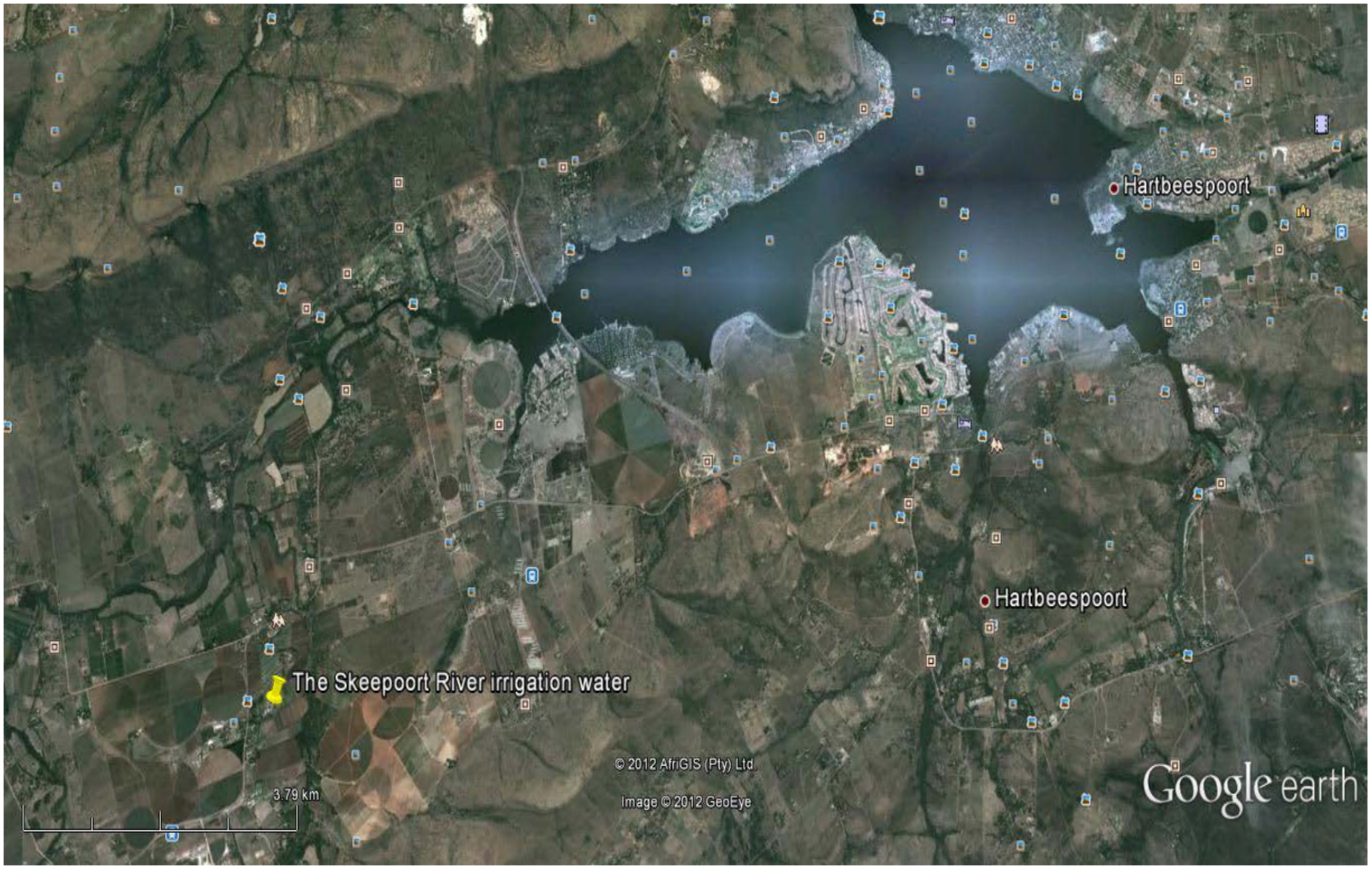

Figure 7: Sampling location for irrigation water from the Skeerpoort river (Source: Google Earth). Accessed: 20/12/12. The Skeerpoort river provides irrigation water for growing fresh produce and the sampling site was downstream the Hartbeespoort dam, a major tourist location with an urban settlement

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PHYSICO-CHEMICAL PARAMETERS

Temperature and pH (Check temp 1, Hana instruments Inc, Rhode Island, USA) of each water sample was measured.

MICROBIOLOGICAL ANALYSIS

Microbiological analysis involved screening for bacterial contaminants (Table 2) in irrigation water from the Loskop canal, the Skeerpoort river and lettuce irrigated with water from the Skeerpoort river.

CHARACTERIZATION AND IDENTIFICATION OF AEROBIC BACTERIA

For each irrigation water and lettuce sample analysed, 3 colonies were randomly picked from the highest dilution standard plate count agar petri-dish. Gram stain and catalase test were done. Ninety (90) bacterial isolates were identified: the Loskop canal (30), the Skeerpoort river (30) and lettuce irrigated with water from the Skeerpoort river (30). Isolates were identified to species level with the Omnilog® data collection software identification system version 2.1 (Biolog Inc. Hayward California).

STATISTICAL ANALYSIS

Analyses of variance was performed using Statistica© software for Windows version 10 (Statsoft Inc, Tulsa, Okalahoma, USA, 2011) to test for significant differences in physiochemical and microbiological quality at 95% confidence interval between the two water sources and irrigation water from Skeerpoort river and irrigated lettuce.

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Table 2: Methodology for detection of bacterial organisms in irrigation water and on lettuce

Organism	Media	Temperature/Time of incubation	Company	*Reference
Aerobic colony count (ACC)	Standard Plate Count Agar	30 °C for 48 to 72 h	(Oxoid Ltd, Basingstoke Hampshire, UK)	SABS ISO 4833 (1991)
Aerobic spore formers (ASF)	Trypticase Soy Agar	35 °C for 48 h	(Biolab Diagnostics (Pty) Ltd, Wadeville Gauteng, South Africa)	(MFLP-44) (Health Canada,
Anaerobic spore formers (AnSF)	Trypticase Soy Agar	35 °C for 48 h	(Biolab)	1998)
Total coliforms	Lauryl Tryptose (LST) Broth	35 °C for 24 to 48h	(Oxoid)	MFHB19 (Health Canada, 2002)
	Brilliant Green Bile 2% Broth (BGLB)	35 °C for 24 to 48h	(Oxoid)	
Faecal coliforms	E.C Broth	35 °C for 24 to 48h	(Oxoid)	
E. coli	Eosin Methylene Blue Agar (Levine) (EMB)	35 °C for 24h	(Oxoid) (Oxoid)	MFHB19 (Health Canada, 2002)
	E. coli/chromogenic Medium	35 °C for 18 to 24h		
	Agar Chrom ID ™ 0157 H7 (O157 ID-F)	35 °C for 18 to 24h	(BioMerieux, Marcyl-l' Etoile, France)	

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Intestinal enterococci	Slanetz and Bartley Medium Bile Esculin Agar	35°C for 44h 44°C for 2h	(Oxoid) (Oxoid)	(SANS ISO 7899-2) (2004)
S. aureus	Baird-Parker Agar	35°C for 48h	(Oxoid)	SABS ISO 6888-1 (1999)
§*Salmonella* spp	Brilliance™ Salmonella Agar Base	35°C for 24 to 48h	(Oxoid)	SABS ISO 6579 (2003)
	Rapid Salmonella	35°C for 24 to 48h	(Biorad, Marnes-la-Coquette France)	
†*L. monocytogenes*	Listeria Selective Agar (Oxford formulation)	35°C for 48 h	(Oxoid)	SABS ISO 11290-1 (1996)
	Listeria Selective Agar (Palcam Selective Supplement)	35°C for 48 h	(Oxoid)	

§-Final confirmation of presumptive *Salmonella* spp was by serotyping at the Agricultural Research Council (ARC)-Ondertespoort, Pretoria, South Africa.

†-Final confirmation of presumptive *L. monocytogenes* was done using Omnilog® Data Collection Software Identification System Version 2.1 (BiologInc.Hayward, California)

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3.2.3 RESULTS

PHYSICO-CHEMICAL QUALITY INDICATORS FOR IRRIGATION WATER FROM THE LOSKOP CANAL AND THE SKEERPOORT RIVER

The pH ranged from 5.4 to 9.9 and 7.9 to 9.2 in the Loskop canal and the Skeerpoort river respectively. The pH in the Loskop canal exceeded national guidelines set by DWAF (DWAF, 1996) for irrigation water twice during the 10 month study. Water temperature in the Loskop canal ranged from 8.5 to 20.5°C with the lowest and highest noted in September and February respectively. Temperature in the Skeerpoort river ranged from 10.7 to 26.0°C with the lowest and highest noted in May and January respectively. Higher mean monthly rainfall was noted in areas around the Skeerpoort river (74.7mm) than the Loskop canal (0.1mm) (Personal communication: Lucky Dlamini, South African Weather Service, July 2012).

BACTERIOLOGICAL QUALITY OF IRRIGATION WATER FROM THE LOSKOP CANAL AND THE SKEERPOORT RIVER

Mean ACC in the Loskop canal and the Skeerpoort river were 3.2 and 3.4 $\log_{10}$cfu/ml respectively (Table 3). Mean AnSF and ASF ranged from 1.8 to 2.5 $\log_{10}$cfu/ml in both water sources. However, high counts (5.1 $\log_{10}$cfu/ml) of ASFs were noted in the Loskop canal in April and October (Figure 8). Additionally high counts of AnSF (5.3 $\log_{10}$cfu/ml) were noted in June and October in the Skeerpoort river (Figure 9). Low mean FC (1.3 $\log_{10}$cfu/ml) were noted in the Loskop canal and the Skeerpoort river (Table 3). FC surpassed national guidelines for irrigation water once (March) in the Skeerpoort river (Figure 9).

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Table 3: Physico-chemical parameters, bacterial counts and incidence of bacterial contaminants in irrigation water from the Loskop canal, the Skeerpoort river and lettuce irrigated with water from the Skeerpoort river for samples (n=30) collected monthly over 10 months

				Indicator Parameters (log_{10} cfu/ml, g)						% samples positive for bacterial contaminants	
	No. of samples	Temp (˚C)	pH	Aerobic Colony Count	Anaerobic Spore Formers	Aerobic Spore Formers	*S. aureus*	IE	FC	*E. coli*	*Salmonella* spp.
Loskop canal[1]	10	15.4±3.7[a]	7.4±1.1[a]	3.2 ±0.7[a]	1.8±1.9[a]	2.1±2.1[a]	0.9±1.2[a]	0.1 ±0.2[a]	1.3±1.0[a]	40	10
Skeerpoort River	10	18.2±6.2[a]	8.4±0.4[a]	3.4±0.6[a]	2.5±1.8[a]	2.5 ± 1.2[a]	0.5 ± 0.8[a]	0.1 ±0.4[a]	1.3 ±1.1[a]	40	ND
Lettuce	10	ND	ND	5.9±0.7[b]	3.3±2.0[a]	3.6±1.4[a]	3.0±1.2[b]	1.2±1.6[a]	1.9±2.1[a]	30	ND
Average	20 (20)	16.8±2.0	7.9±0.7	3.3±0.1 (4.9±1.8)	2.2±0.5 (2.9±0.6)	2.3±0.3 (3.1±0.8)	0.7±0.3 (1.8±1.8)	0.1±0.0 (0.7±0.8)	1.3±0.0 (1.6±0.4)	40 (35)	5

FC- Faecal coliforms (log_{10} MPN/100ml, g)
S. aureus- *Staphylococcus aureus*
IE - Intestinal enterococci
[1] *Salmonella enterica* subsp *salamae* was isolated from the Loskop canal
± Standard deviation
ND- not determined
Values with different letters in same column significantly different ($p \leq 0.05$)
Average for bacterial indicators between irrigation water from the Skeerpoort river and irrigated lettuce in parentheses

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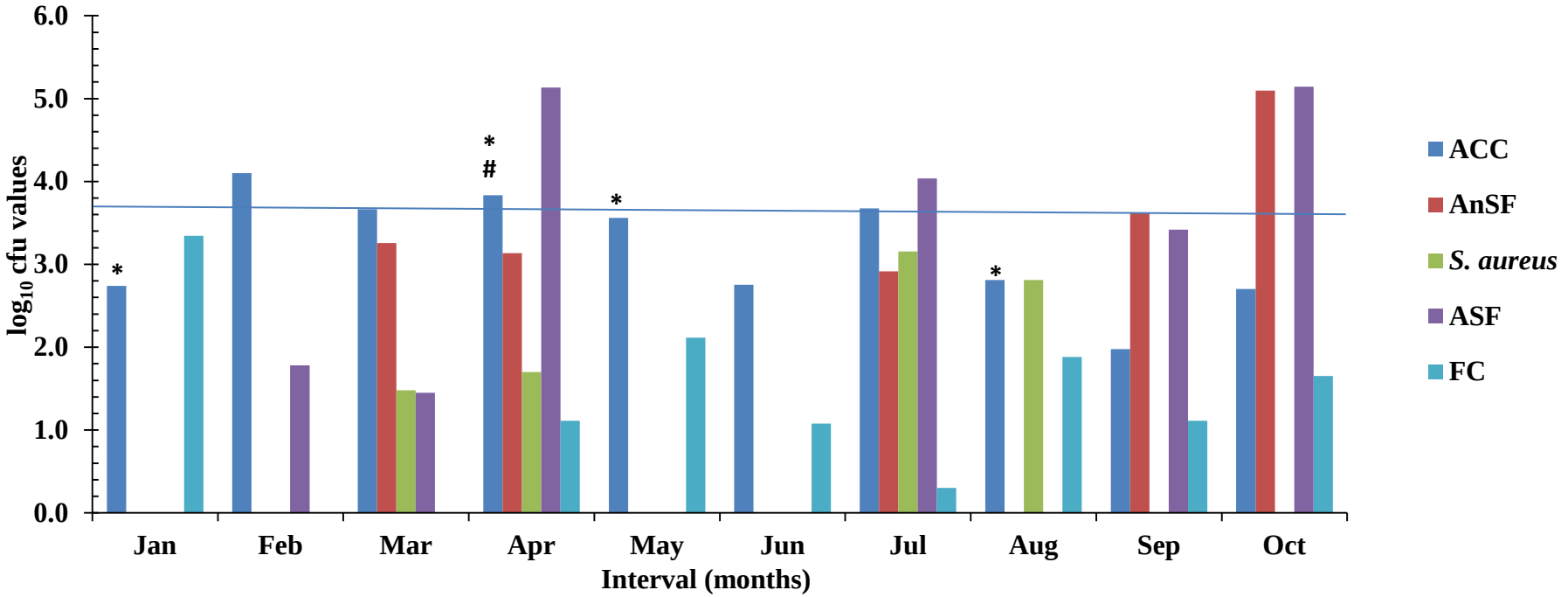

Figure 8: Change of bacterial counts and incidence of *E. coli* and *Salmonella* spp with time in irrigation water from the Loskop canal for samples (n=10) collected monthly over 10 months. (*) Interval positive for *E. coli*, (#) Interval positive for *Salmonella enterica* subsp *salamae*. Aerobic colony count (ACC), Anaerobic spore formers (AnSF), Aerobic spore formers (ASF), *Staphylococcus aureus* (*S. aureus*) and Faecal coliforms (FC: $\log_{10}$ MPN/100ml). Horizontal line represents upper limit for faecal coliforms in irrigation water (DWAF, 1996)

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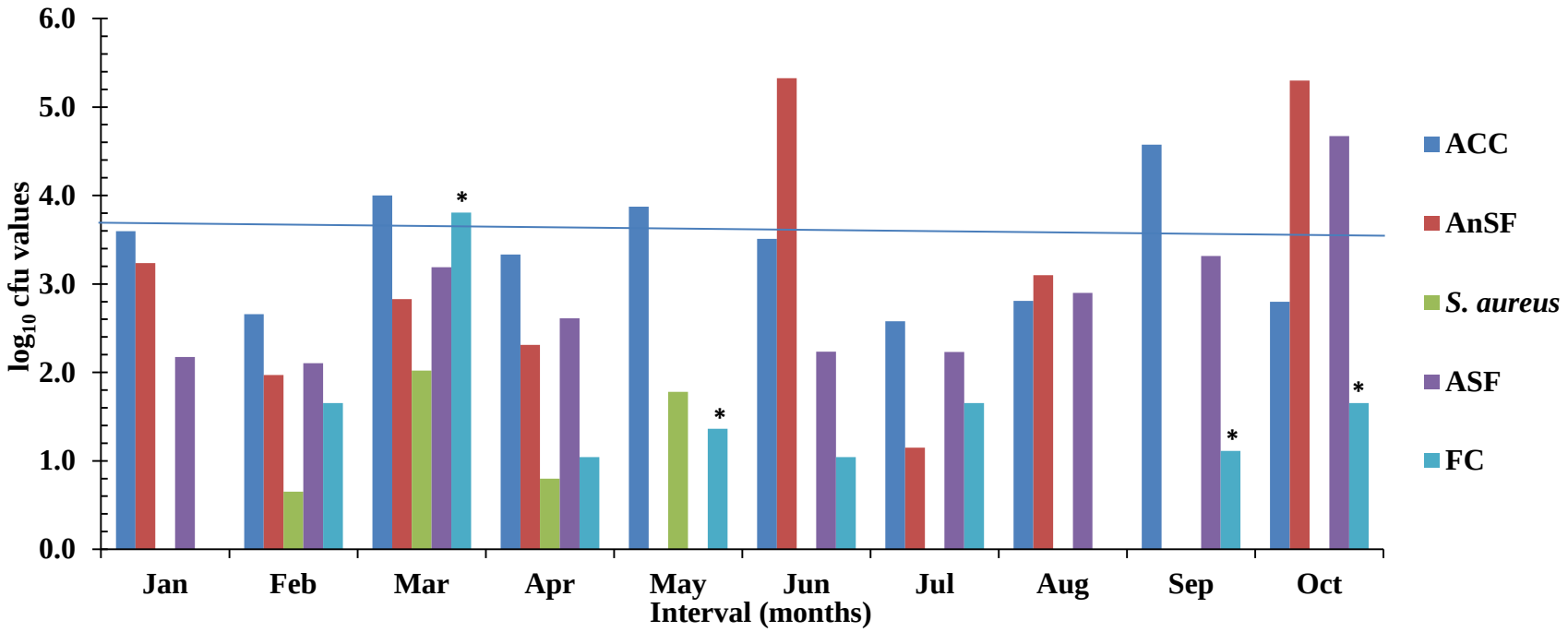

Figure 9: Change of bacterial counts and incidence of *E. coli* with time in irrigation water from the Skeerpoort river for samples (n=10) collected monthly over 10 months. (*) Interval positive for *E. coli*. Aerobic colony count (ACC), Anaerobic spore formers (AnSF), Aerobic spore formers (ASF), *Staphylococcus aureus* (*S. aureus*) and Faecal coliforms (FC: (log_{10}MPN/100ml)). Horizontal line represents upper limit for faecal coliforms in irrigation water (DWAF, 1996)

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Counts of IE ranged from 0.1 to 0.4 $\log_{10}$cfu/ml in the Loskop canal and the Skeerpoort river (Table 3). Prevalence of *E. coli* was 40% at both irrigation water sites (Figures 8 & 9). *E. coli* was isolated from the Loskop canal and the Skeerpoort river in months when the highest faecal coliform counts were recorded (Figures 8 & 9). Additionally *E. coli* O157:H7 was isolated from both irrigation water sites. *S. aureus* was noted on few months at both irrigation water sites. The pathogen was noted on 3 (the Skeerpoort river) and 4 (Loskop canal) months during the ten month study (Figures 8 & 9). However when *S. aureus* was noted, it ranged from 1.6 to 3.0 log10cfu/ml in the Loskop canal and 0.6 to 2.0 log10cfu/ml in the Skeerpoort river (Figures 8 and 9). *Salmonella enterica* subsp *salamae* (typed as *Salmonella* II 13, 22, 23) was isolated from the Loskop canal (Table 3).

BACTERIAL COMPOSITION OF IRRIGATION WATER FROM THE LOSKOP CANAL AND THE SKEERPOORT RIVER

Seven bacterial genera/species were isolated from the Loskop canal. They included *Bacillus* spp, *Enterobacter* spp, *E. coli*, *Klebsiella* spp, *Kluyvera ascorbata*, *Enterococcus gallinarum* and *Serratia marcescens* ss. *marcescens* (Table 4). Eight genera/species were isolated from the Skeerpoort river. They included *E. coli*, *Bacillus* spp, *Enterobacter* spp, *Klebsiella* spp, *Raoultella* spp, *Burkholderia* spp, *Buttiauxella* spp and *Salmonella* spp (Table 4). *Bacillus* spp, *E. coli and Enterobacter* spp were the most prevalent bacteria in the Loskop canal and the Skeerpoort river (Figures 11 & 12).

E. coli was the most prevalent species isolated in the winter and *Bacillus* spp in summer at both irrigation water sites. Low prevalence (3.4%) was noted for *Kluyvera ascorbata*, *K. oxytoca*, *Enterococcus gallinarum*, *K. pneumoniae* subsp. *pneumonia* and *S. marcescens* subsp. *marcescens* in the Loskop canal (Figure 11). Similarly low prevalence (3.3%) of *Salmonella* spp, *Klebsiella* spp, *Raoultella* spp, *Burkholderia* spp, *Buttiauxella* spp were noted in the Skeerpoort river (Figure 12).

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Table 4: Prevalence of predominant bacteria in irrigation water from the Loskop canal and the Skeerpoort river for isolates (n=60) collected monthly over 10 months

Month	1		2		3		4		5		6		7		8		9		10		
Sample	L C	S R	L C	S R	L C	S R	L C	S R	L C	S R	L C	S R	L C	S R	L C	S R	L C	S R	L C	S R	
Percentage isolates																					Total number of isolates
E. coli	33	–	33	–	–	–	–	–	–	67	67	100	–	33	33	–	67	100	–	67	18
Bacillus spp	33	33	33	100	100	33	33	33	33	33	33	–	–	–	–	33	–	–	67	–	18
Enterobacter spp	33	33	33	–	–	–	33	67	33	–	–	–	67	33	33	–	33	–	–	33	13
Klebsiella spp	–	–	–	–	–	67	–	–	33	–	–	–	–	–	33	–	–	–	–	–	4
Enterococcus spp	–	–	–	–	–	–	–	–	–	–	–	–	33	–	–	–	–	–	–	–	1
Salmonella spp	–	33	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
Serratia spp	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	33	–	1
Raoultella spp	–	–	–	–	–	–	–	–	–	–	–	–	–	33	–	–	–	–	–	–	1
Klyuvera ascorbata	–	–	–	–	–	–	33	–	–	–	–	–	–	–	–	–	–	–	–	–	1
Burkholderia caprophylil	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	33	–	–	–	–	1
Buttiaxella agrestis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	33	–	–	–	–	1
Total number of isolates	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	60
ACC (log_{10} cfu/ml)	2.7	3.6	4.1	2.7	3.7	4.0	3.8	3.3	3.6	3.9	2.8	3.5	3.7	2.6	2.8	2.8	2.0	4.6	2.7	2.8	

L C, the Loskop canal; S R, the Skeerpoort river; -, Not determined; ACC, aerobic colony count

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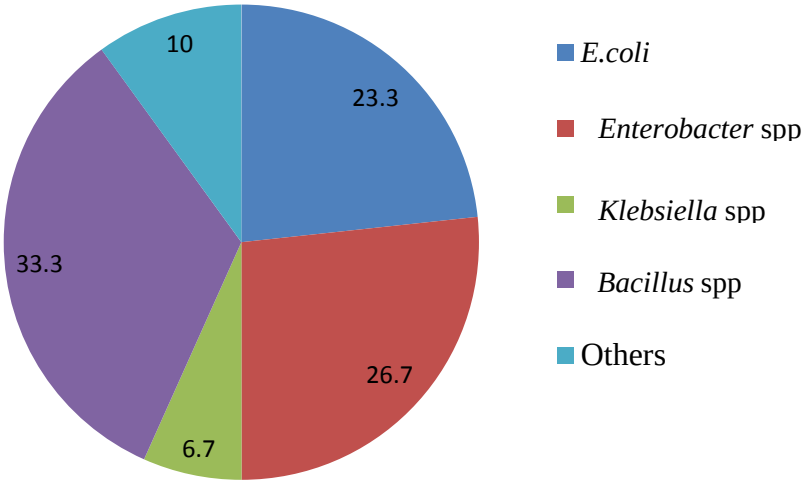

Figure 11: Prevalence of predominant bacteria (n=30) in irrigation water from the Loskop canal for samples (n=10) collected monthly over 10 months. Others: *Kluyvera ascorbata* and *Serratia marcescens* ss. *marcescens*

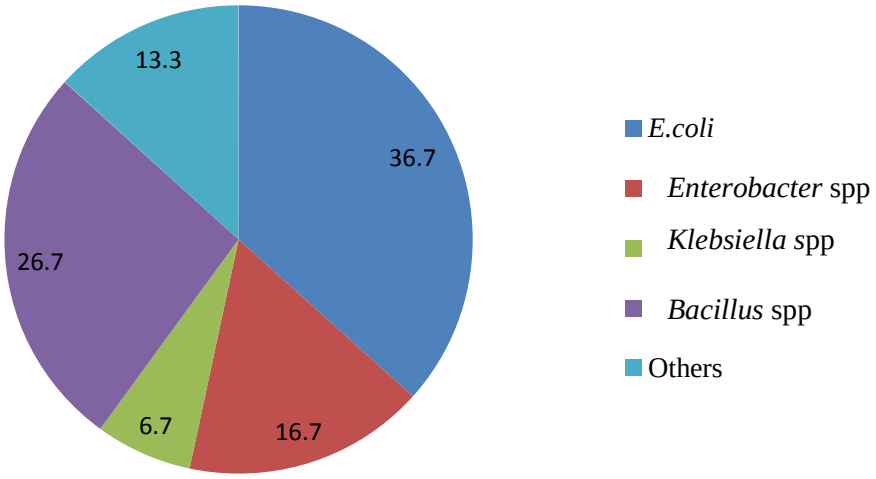

Figure 12: Prevalence of predominant bacteria (n=30) in irrigation water from the Skeerpoort river for samples (n=10) collected monthly over 10 months. Others: *Raoultella terringena*, *Burkholderia caprophylil*, *Buttiauxella agrestis* and *Salmonella enterica*

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BACTERIOLOGICAL QUALITY OF IRRIGATION WATER FROM THE SKEERPOORT RIVER AND IRRIGATED LETTUCE

Higher mean ACC were noted on lettuce (5.9 $\log_{10}$ cfu/g) than irrigation water from the Skeerpoort river (3.4 $\log_{10}$ cfu/ml) (Table 3). There was a significant difference ($p \leq 0.05$) between ACC of irrigation water from the Skeerpoort river and lettuce as well as over the sampling months. The highest ACC on lettuce were noted in August (7.3 $\log_{10}$ cfu/g) (Figure 10). Low mean counts of AnSFs and ASFs were noted in irrigation water from the Skeerpoort river and irrigated lettuce (Table 3).

Low mean FC counts were noted in irrigation water and on lettuce (1.9 $\log_{10}$ MPN) (Table 3). However sporadic spikes in FC counts above the mean levels were noted on irrigated lettuce on 3 of the 10 months during the study (Figure 10). The highest FC counts in irrigation water and on lettuce were both noted during summer (Figures 9 & 10). Mean IE counts on lettuce were low (Table 3). *E. coli* was isolated from irrigation water and on lettuce when high faecal coliform counts were also noted (Figures 9 & 10). Counts for *S. aureus* were significantly ($p \leq 0.05$) higher on lettuce than irrigation water in the Skeerpoort river (Table 3). Additionally counts of *S. aureus* on lettuce and in irrigation water from the Skeerpoort differed significantly ($p \leq 0.05$) over sampling months.

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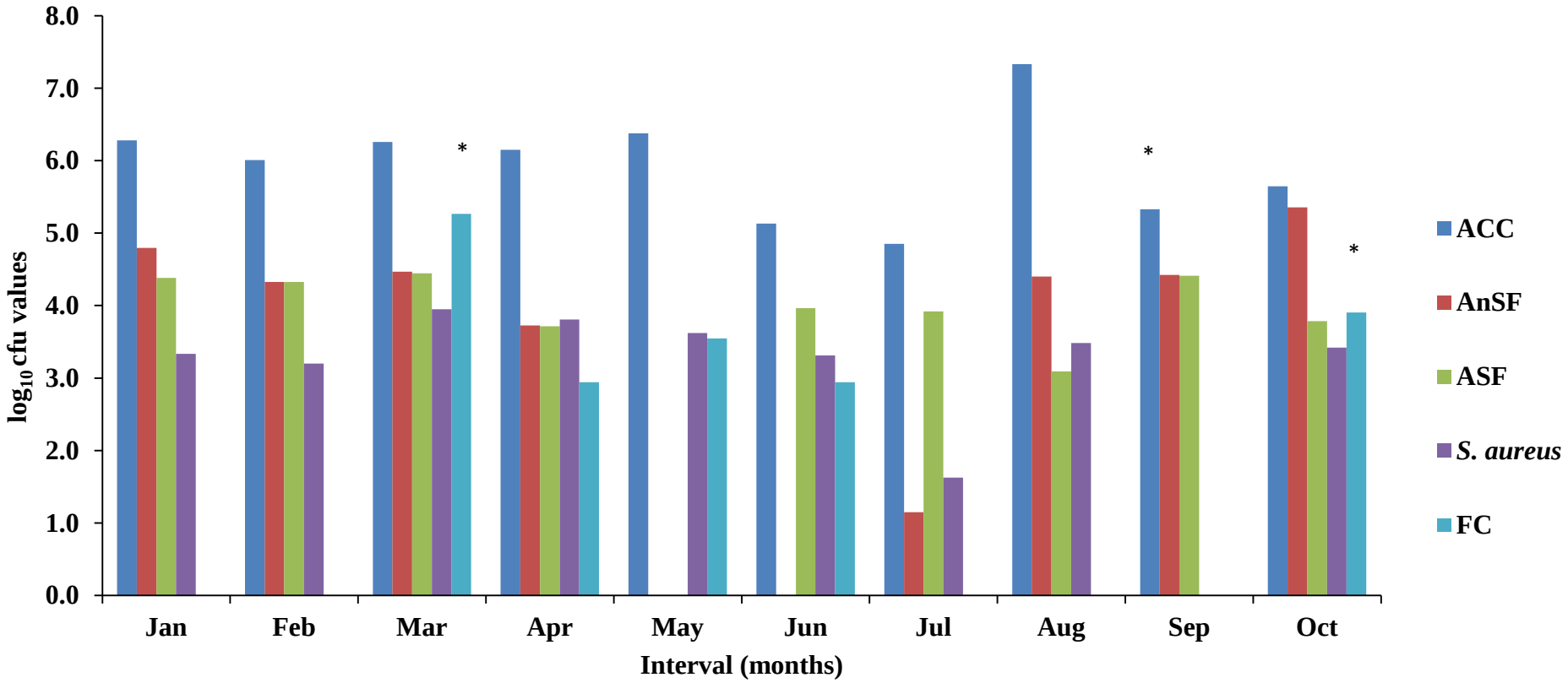

Figure 10: Change in bacterial counts and incidence of *E. coli* on lettuce irrigated with water from the Skeerpoort river for samples (n=10) collected monthly over 10 months. (*) Interval positive for *E. coli*. Aerobic colony count (ACC), Anaerobic spore formers (AnSF), Aerobic spore formers (ASF), *Staphylococcus aureus* (*S. aureus*) and Faecal coliforms (FC:$\log_{10}$ MPN/g)

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BACTERIAL COMPOSITION OF IRRIGATION WATER FROM THE SKEERPOORT RIVER AND IRRIGATED LETTUCE

Bacterial species were more diverse on irrigated lettuce than in the irrigation water from the Skeerpoort river (Table 5). Eleven genera were isolated on lettuce. They included *E. coli*, *Bacillus* spp, *Enterobacter* spp, *Klebsiella* spp, *Leclercia adecarboxylata*, *Staphylococcus kloosi*, *Brevibacillus borstelensis*, *Exiguobacterium undae*, *R. planticola/ornithinolytica*, *E. gallinarum*, *S. marcescens* ss. *Mercescens* (Table 5). Similarly as noted with water from the Skeerpoort river, *E. coli*, *Bacillus* spp and *Enterobacter* spp were the most prevalent bacterial genera isolated from lettuce (Figure 13). *E. coli* was the most prevalent isolated species from lettuce in winter. Prevalence of *Enterobacter* spp was lower on lettuce than in irrigation water (Figures 12 & 13).

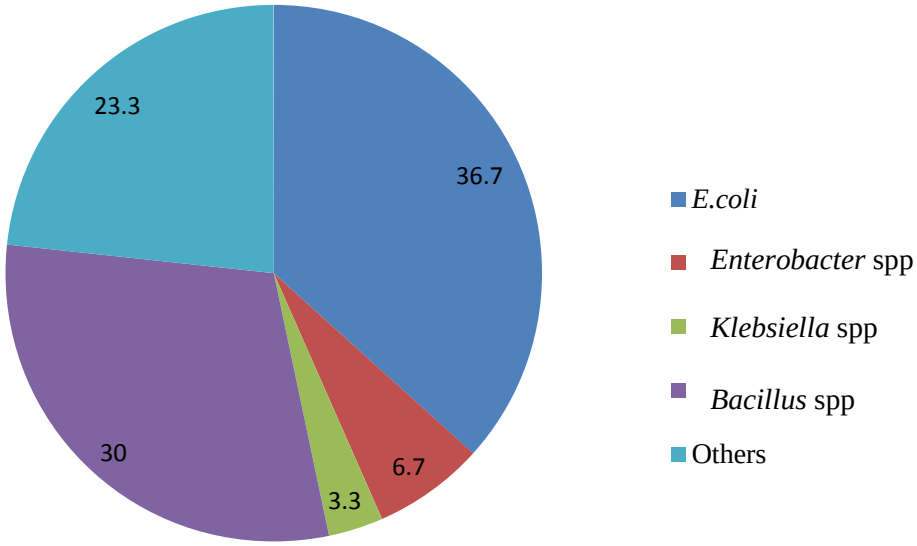

Figure 13: Prevalence of predominant bacteria (n=30) isolated on lettuce irrigated with water from the Skeerpoort river for samples (n=10) collected monthly over 10 months. Others: *Leclercia adecarboxylata*, *Staphylococcus kloosi*, *Brevibacillus borstelensis*, *Exiguobacterium undae*, *Raoultella planticola/ornithinolytica*, *Enterococcus gallinarum* and *Serratia marcescens* ss. *mercescens*

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Table 5: Prevalence of predominant bacteria in irrigation water from the Skeerpoort river and on lettuce irrigated with water from the Skeerpoort river for isolates (n=60) collected monthly over 10 months

Month	1		2		3		4		5		6		7		8		9		10		
Sample	SR	L	SR	L	SR	L	SR	L	SR	L	SR	L	SR	L	SR	L	SR	L	SR	L	Total number of isolates
Percentage isolates																					
E. coli	–	–	–	–	–	33	–	–	67	67	100	67	33	33	–	–	100	100	67	67	22
Bacillus spp	33	67	100	33	33	–	33	33	33	33	–	–	–	33	33	100	–	–	–	–	17
Enterobacter spp	33	–	–	33	–	33	67	–	–	–	–	–	33	–	–	–	–	–	–	–	7
Klebsiella spp	–	–	–	–	67	33	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3
Enterococcus spp	–	–	–	–	–	–	–	–	–	–	–	–	–	33	–	–	–	–	–	–	1
Salmonella spp	33	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
Serratia spp	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	33	1
Raoultella spp	–	–	–	–	–	–	–	–	–	–	–	33	33	–	–	–	–	–	–	–	2
Leclercia adecarboxylata	–	33	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
Brevibacillus borstelensis	–	–	–	–	–	–	–	33	–	–	–	–	–	–	–	–	–	–	–	–	1
Exiguobacterium undae	–	–	–	–	–	–	–	33	–	–	–	–	–	–	–	–	–	–	–	–	1
Staphylococcus kloosi	–	–	–	33	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
Bautiaxella agrestis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	33	–	–	–	–	–	1
Burkholderia caprophylil	–	–	–	–	–	–	–	–	–	–	–	–	–	–	33	–	–	–	–	–	1
Total number of isolates	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	60
ACC (log_{10} cfu/ml)	3.6	6.3	2.7	6.0	4.0	6.3	3.3	6.1	3.9	6.4	3.5	5.1	2.6	4.9	2.8	7.3	4.6	5.3	2.8	5.6	

S R, the Skeerpoort river; L, lettuce irrigated with water from the Skeerpoort river ; -, Not determined; ACC, aerobic colony count

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3.2.4 DISCUSSION

The fluctuations noted in water temperature from the Loskop canal and the Skeerpoort river were due to seasonal changes resulting from summer and winter. Such fluctuations may have played a role in selecting for *E. coli* and *Bacillus* spp. Temperature has been noted as a major factor influencing proliferation of bacterial pathogens (Pachepsky *et al*., 2011). The pH fluctuations out of the recommended range noted in the Loskop canal may have resulted from the surrounding land use practices. There are defunct flooded underground coal mines which contribute to pollution in water sources of the upper Olifants river catchment located upstream the Loskop canal (CSIR, 2010). Therefore effects of mining such as acid drainage and industrial effluents may have caused changes in pH of water in the Loskop canal. Similar sporadic pH values were previously noted in the Loskop canal (Ijabadeniyi *et al*., 2011b). Sporadic changes in ACC, ASF and AnSF in the Loskop canal and the Skeerpoort river may have been due to isolated events of pollution at different times.

Bacterial indicator counts may vary as a result of availability of nutrient growth requirements, settling to sediment, chemical reactions, decay or due to seasonal changes (Pachepsky *et al*., 2011). Low mean counts for bacterial indicators in the Loskop canal may have been due to the fencing around the canal, concrete flooring and side walls which might have reduced bacterial contamination from extraneous sources such as animals and run-off. Use of a reservoir dam for collecting irrigation water in the Skeerpoort river may have protected it from contamination sources commonly associated with free flowing rivers such as runoff, animal faecal material, domestic sewage and industrial effluent. Low and sporadic counts of ACC, ASF and ASF were previously noted in the Loskop canal (Ijabadeniyi *et al*., 2011b).

The higher mean ACC, *S. aureus* counts and diversity of bacterial species noted on lettuce than irrigation water from the Skeerpoort river was probably because the lettuce phyllosphere provides a more suitable environment for bacterial proliferation compared to flowing irrigation water. The lettuce phyllosphere has a rough surface and external openings such as the stomata that can provide points for attachment of bacterial pathogens (Aruscavage *et al*., 2008). Additionally leaf surfaces even with the slightest abrasion undergo microscopic damage that causes leaching out of nutrients such as simple sugars and organic acids that can be metabolized by bacterial pathogens aiding their proliferation within a secondary habitat (Aruscavage *et al*., 2008). Furthermore the lettuce phyllosphere has epiphytic micro flora which can form biofilms with bacterial pathogens encasing them in an exo-polysaccharide matrix that provides protection against adverse environmental conditions (Matthews, 2009).

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Contamination with *S. aureus*, a pathogen normally found in the nasal cavity may have been thorough contact with human handlers during pre-harvest field operations. Additionally contact of lettuce with animals in the field and soil may have been a source of *S. aureus* contamination (Pachepsky *et al*., 2011).

Counts for bacterial indicators on lettuce that followed no particular pattern in relation to irrigation water coupled with wider diversity of isolated bacterial species suggested contamination from other environmental sources. This is may have been due to the study being carried out under field conditions. Lettuce grows in close proximity to the ground and therefore soil may have been a source of bacterial contamination especially due to splashes resulting from spray irrigation which was the mode of irrigation used. Additionally the accumulation of bacteria on lettuce resulting from contamination from irrigation water as well as other environmental sources may have resulted in higher numbers of bacterial contaminants on lettuce. Bacterial indicator counts that followed no particular trend were noted in irrigation water and on fresh produce grown under field conditions (Ackermann, 2010; Ijabadeniyi *et al*., 2011b).

The high FC noted during summer when high rainfall was also noted suggests a positive relationship between faecal contamination and rainfall. This was possibly due to larger volume of runoff generated that increased contamination within the water source as well as the churning of sediment (Korajkic *et al*., 2010). High prevalence of *E. coli* noted at the two sites is indicative of possible faecal contamination and ability of the pathogen to stay viable in irrigation water during winter (Islam *et al*., 2004). This may increase risk of pathogenic bacterial possible transfer and proliferation on irrigated produce. *E. coli* was isolated from irrigation water and on fresh produce irrigated with water from the Loskop canal (Ijabadeniyi *et al*., 2011b) and the Skeerpoort river (Duhain, 2011).

Predominance of *E. coli* during winter at both sites and on lettuce suggests adaptation to low environmental temperature compared to other bacterial species isolated. *E. coli* has been suggested to apply a dual regulation system enabling selection and production of scarce metabolic requirements which assist and maintain growth in secondary environments such as water and the plant phyllosphere (Seurinck *et al*., 2005). Additionally *E. coli* have petrichous pili-flagella (Welch, 2006) that aid movement in liquid environments, an adaptation that may enhance survival by enabling transfer to nutrient rich areas. These adaptations may help *E. coli* stay viable in irrigation water increasing likelihood of contaminating irrigated produce.

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High prevalence of *E. coli* has previously been noted at lower temperatures of 8˚C (Berry and Wells, 2010). The Department of Health (DoH) in South Africa recommends absence of *E. coli* on fresh produce (DoH, 2006), therefore presence of the microorganism may signal unsuitability for human consumption due to risk of foodborne disease.

High prevalence of *Bacillus* spp noted in the Loskop canal and the Skeerpoort river could be attributed to its ubiquitous nature. *Bacillus* spp is widely distributed in sediment, run-off from soil and decaying matter (Ells and Hansen, 2006). All of which usually end up in irrigation water sources as a result of environmental, human and/or animal contamination. The high prevalence of *Bacillus* spp during summer as opposed to winter maybe due to warmer temperature that favours spore germination into vegetative cells and hence proliferation. During winter, *Bacillus* spp may revert to spores as a means of protecting itself from unfavourable conditions.

Enterobacter spp and *K. pneumonia* are common opportunistic pathogens. *K. pneumonia* proliferates in the human intestines and is found in faeces (CDC, 2012b) therefore its presence may indicate possible faecal contamination at both irrigation water sites. *K. pneumonia* has been isolated from irrigation water and lettuce (Olayemi, 1997) as well as from raw lettuce on sale in markets (Puspanadan *et al*., 2012). *K. pneumonia* spp is usually associated with healthcare associated infections, wounds or surgical site infections and meningitis in individuals with compromised immunity such as infants, the ill and aged (CDC, 2012b). *K. pneumonia* has been noted as a cause of infections among new born babies in South Africa (Ballot *et al*., 2012) and other developing countries (Zaidi *et al*., 2009; Decré *et al*., 2011) with sources of infection linked more to the environment than maternal hygiene (Zaidi *et al*., 2009). High prevalence of *Enterobacter* spp (Zamxaka *et al*., 2004; Lin and Biyela, 2005; Lötter, 2010) and *Klebsiella* spp (Samie *et al*., 2010) have been noted in South African water sources. The isolation of similar bacterial indicators and foodborne pathogens from irrigation water in the Skeerpoort river and on lettuce suggests that irrigation water may have been a source of contamination on lettuce and therefore irrigated produce from these sites may pose a food safety and health risk to consumers.

3.2.5 CONCLUSION

Irrigation water in the Loskop canal and the Skeerpoort river is contaminated with bacterial foodborne pathogens a scenario that may reflect on the bacteriological quality of other irrigation water sources in the country. Additionally lettuce irrigated with water from the

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Skeerpoort river was contaminated with bacterial pathogens and may pose risk of causing illness to consumers of such produce. This may compromise food safety and public health as well as future profitability of the fresh produce market in South Africa in case foodborne outbreaks occur. Therefore limiting pollution of irrigation water sources by identifying land use practices causing contamination is necessary in order to maintain clean and safe irrigation water and subsequently irrigated fresh produce. Seasonality as well as rainfall precipitation may select for bacterial pathogens within surface irrigation water sources and thereby increasing risk of transfer onto irrigated produce. Therefore further investigations into how these factors affect proliferation of bacterial pathogens within the pre-harvest environment may help shed light on how to effectively limit pathogenic bacterial contamination on irrigated fresh produce. This may reduce food safety risks associated with bacterial pathogens on irrigated fresh produce.

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3.3 ANTIBIOTIC RESISTANCE AND VIRULENCE GENES IN *E. COLI* COLLECTED FROM THE LOSKOP CANAL, SKEERPOORT RIVER AND IRRIGATED LETTUCE

ABSTRACT

This study aimed at determining the prevalence of antibiotic resistant and virulent *E. coli* collected from the Loskop canal, the Skeerpoort river and lettuce irrigated with water from the Skeerpoort river. Forty one (41) *E. coli* isolates: (19) the Loskop canal; (12) the Skeerpoort river; (10) lettuce were tested with 11 antibiotics at single concentrations and screened for Shigatoxin 1 (*stx 1*), Shigatoxin 2 (*stx 2*) and intimin (*eae*) genes. Antibiotic resistance was also used as a means of clustering *E. coli* isolated from the 3 sources. Resistance of isolates to at least one antibiotic was 84.2% and 83.3% in Loskop canal and the Skeerpoort river respectively. There was a significant difference ($p \leq 0.05$) in resistance to antibiotics between isolates from the Loskop canal and the Skeerpoort river. Additionally the combined effect of isolate source (irrigation water site) and antibiotics for isolates from the Skeerpoort river was significant ($p \leq 0.05$). From lettuce, 90% of isolates were resistant to at least one antibiotic and resistance significantly differed ($p \leq 0.05$) with isolates from the Skeerpoort river. The highest resistance to single antibiotics in all three samples was to cephalothin and ampicillin. Higher resistance was noted to multiple (more than 2) antibiotics in the Skeerpoort river (33.3%) than Loskop canal (5.3%). Most isolates from the same source showed close relatedness. Close relatedness was shown by isolates in the Loskop canal (10.5%) and the Skeerpoort river (16.7%). From irrigated lettuce 40% of isolates showed close relatedness to isolates from the Skeerpoort river. In the Loskop canal 15.7% and 41.7% of isolates in the Skeerpoort river possessed virulence genes. From lettuce, 20% of isolates possessed virulence genes. In the Loskop canal and from lettuce all isolates with virulence genes were antibiotic resistant while 80% of isolates with virulence genes in the Skeerpoort river were antibiotic resistant. In the Loskop canal 10.5% and 25% of isolates in the Skeerpoort river were positive for *stx1*/*stx2* and *eae*, genes synonymous with Enterohaemorrhagic *E. coli* (EHEC). Results from this study show that *E. coli* from the two irrigation water sources as well as on irrigated lettuce were resistant to antibiotics and potentially infectious. This may increase risk of contaminating irrigated fresh produce during postharvest processing thereby compromising food safety and public health.

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3.3.1 INTRODUCTION

The World Health Organization (WHO) has reported on increasing prevalence of antibiotic resistance among foodborne bacterial pathogens (WHO, 2009). Increase in foodborne outbreaks associated with fresh produce (CDC, 2012a) may be linked to complications resulting from illness caused by antibiotic resistant pathogens (Olaniran *et al*., 2009). Antibiotic resistant pathogens such as *E. coli* pose a risk to public safety (Newell *et al*., 2010) as illness resulting from infection may affect a large section of people causing serious complications and fatality. Antibiotic resistance can be acquired through exchange of mobile genetic fragments on the plasmids of these pathogens (Da Silva and Medonça, 2012) and with the high prevalence of *E. coli* noted at both irrigation sites and on lettuce (phase I) such a scenario may occur. Determination of antibiotic resistance among bacterial pathogens such as *E. coli* isolated from river sources used for irrigation may be useful in accessing the food safety and health risk posed to the public (Lin and Biyela, 2005). High prevalence of antibiotic resistant *E. coli* has been noted in South African river sources (Obi *et al*., 2004; Olaniran *et al*., 2009; Kinge *et al*., 2010).

Antibiotic resistance may provide information on phenotypic relationships existing among environmental *E. coli* which could give an indication on possible sources of contamination (Parveen *et al*., 1997; Vantakaris *et al*., 2006; Kinge *et al*., 2010). Multiple antibiotic resistance (MAR) and antibiotic resistance analysis (ARA) have been used to determine phenotypic characteristics of faecal bacteria such as *E. coli* in addition to indicating possible sources of faecal contamination as part of microbial source tracking (MST) (Olivas and Faulkner, 2008). Determining antibiotic resistance of *E. coli* may provide meaningful assessment of contamination levels in irrigation water helping in the formulation of strategies for better management of irrigation water sources. Although a number of studies in South Africa have reported on the prevalence of foodborne pathogens in irrigation water and fresh produce, few have characterized the virulence factors in the pathogens. Obi *et al*., (2004) and (Olaniran *et al*., 2009) previously noted low prevalence of Shigatoxins in *E. coli* isolated from river sources located in a rural Venda and Durban respectively. This study provides the first characterization of antibiotic resistance and virulence genes in *E. coli* from the Loskop canal (Mpumalanga Province), the Skeerpoort river (North West Province) and on lettuce irrigated with water from the Skeerpoort river. The aim of the study was to compare prevalence of antibiotic resistant and pathogenic *E. coli* in the two irrigation water sources

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and whether irrigation water from the Skeerpoort river was a source of antibiotic resistant and virulent *E. coli* on irrigated lettuce grown under field conditions.

3.3.2 ANTIBIOTIC RESISTANCE OF *E. COLI* ISOLATED FROM THE LOSKOP CANAL, SKEERPOORT RIVER AND IRRIGATED LETTUCE

MATERIALS AND METHODS

BACTERIAL STRAINS

Forty one (41) environmental *E. coli* strains: (19) the Loskop canal; (12) the Skeerpoort river; (10) lettuce irrigated with water from the Skeerpoort river were used in the study. Thirteen (13) of the isolates were isolated from (Eosin Methylene Blue agar (Levine) (EMB) (Oxoid, Ltd, Basingtoke Hampshire, UK) and identified with selective *E. coli*/chromogenic medium (Oxoid, Ltd, Basingtoke Hampshire, UK) and Agar Chrom ID ™ 0157 H7 (O157 ID-F) (BioMerieux, Marcyl-l' Etoile, France). Twenty nine (29) of the isolates were identified using Omnilog® Data Collection Software Identification System Version 2.1 (Biolog Inc. Hayward California).

ANTIBIOTICS AND DISK DIFFUSION TESTING

Eleven (11) antibiotics (Oxoid Ltd, Basingstoke, UK) at single concentrations were selected according to Da Silva *et al.*, (2011). Antimicrobial susceptibility was done using MAR analysis (Vantakaris *et al*., 2006). Antibiotics used included; Amikacin (30µg), Gentamicin (10µg), Chloramphenicol (30µg), Nalidixic Acid (30µg), Norfloxacin (10µg), Neomycin (30µg), Nitrofurantoin (300µg), Amoxicillin (25µg), Ampicillin (10µg), Cephalothin (30µg) and Oxytetracycline (30µg). Antibiotic susceptibility testing was determined by disc diffusion method using Mueller-Hinton agar (Oxoid).

STATISTICAL ANALYSIS

Analyses of variance was performed to test for significant differences in antibiotic resistance patterns for *E. coli* isolated from the Loskop canal, Skeerpoort river and lettuce at 95% confidence interval. For numerical classification, antibiotic measurements (mm) were used to determine inter-isolate relationships by weighted pair-group average euclidean distances (Sneath and Sokal, 1973). Clusters were defined at euclidean distance of 2.0. All analyses were done with Statistica© software for Windows version 10 (Statsoft Inc, Tulsa, 2011).

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3.3.3 VIRULENCE GENES IN *E. COLI* ISOLATED FROM THE LOSKOP CANAL, SKEERPOORT RIVER AND LETTUCE

MATERIALS AND METHODS

The same *E. coli* isolates used for antibiotic resistance were tested for the following virulence genes: Shigatoxin 1 (*stx 1*), Shigatoxin 2 (*stx 2*) and intimin (*eae*) (Bio-rad). The thermocycler (C1000 Touch ThermalCycler CFX96^{TM} Real Time System) (Bio-Rad) and software (CFX Manager IDE) (Bio-rad) were set up for analysis using iQ-Check™ STEC VirX catalogue # 357-8139 (Bio-rad).

3.3.4 RESULTS

ANTIBIOTIC RESISTANCE AND VIRULENCE IN *E. COLI* FROM THE LOSKOP CANAL AND THE SKEERPOORT RIVER

Isolates from the Loskop canal were resistant to nitrofurantoin, ampicillin, nalidixic acid, gentamicin, oxytetracycline, amikacin, cephalothin, neomycin and amoxicillin while isolates from the Skeerpoort river showed resistance to nalidixic acid, norfloxacin, neomycin, amoxycillin, ampicillin, cephalothin and oxytetracycline. Resistance of isolates to at least one antibiotic was 84.2% and 83.3% in the Loskop canal and in the Skeerpoort river respectively. Highest resistance to single antibiotics in both water sites was to cephalothin and ampicillin (Table 6).

The percentage of isolates resistant to more than one antibiotic in the Loskop canal and the Skeerpoort river was 42.1% and 50% respectively. However, only 5.3% of isolates in the Loskop canal compared to 33.3% in the Skeerpoort river were resistant to more than two antibiotics. There was a significant difference ($p \leq 0.05$) in resistance to antibiotics among isolates from the Loskop canal and the Skeerpoort river. All isolates were susceptible to norfloxacin and chloramphenicol in the Loskop canal. All isolates in the Skeerpoort river were susceptible to amikacin, gentamicin, chloramphenicol and nitrofurantoin. In the Loskop canal 8 antibiotic resistant patterns were noted while 6 were noted in the Skeerpoort river (Table 7). More isolates from the Skeerpoort river showed resistance to multiple antibiotics than in the Loskop canal (Table 7).

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Table 6: Prevalence of antibiotic resistant *E. coli* collected over 10 months in irrigation water from the Loskop canal and the Skeerpoort river

	% of Resistant isolates	
Antibiotic	Loskop canal (n=19)	Skeerpoort river (n=12)
Amikacin, 30µg	5.3	ND
Gentamicin, 10µg	5.3	ND
Chloramphenicol,30µg	ND	ND
Nalidixic acid, 30µg	5.3	16.7
Norfloxacin, 10µg	ND	8.3
Neomycin, 30µg	5.3	8.3
Nitroforantoin,300µg	10.5	ND
Amoxycillin, 25µg	5.3	16.7
Ampicillin, 10µg	21.1	50
Cephalothin, 30µg	73.7	50
Oxytetracyline, 30µg	5.3	25

ND - No resistance

Most of the isolates from the same irrigation water source showed close phenotypic relatedness based on resistance to the antibiotics (Figure 14). However 10.5% and 16.7% of isolates from the Loskop canal (7 &18) and the Skeerpoort river (f & d) showed close phenotypic relatedness based on antibiotic resistance respectively (Figure 14).

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Table 7: Multiple resistances to antibiotics in *E. coli* isolated over 10 months from irrigation water in the Loskop canal and the Skeerpoort river

	% of isolates with indicated resistance pattern	
Pattern of antibiotic resistance	Loskop canal (n=19)	Skeerpoort river (n=12)
KF-AK	5.3	ND
KF-AMP	15.8	8.3
KF-F	5.3	ND
KF-OT	5.3	8.3
KF-CN	5.3	ND
F-OT	5.3	ND
AMP-N	5.3	ND
AMP-KF-AML-F	5.3	ND
KF-AMP-NA	ND	8.3
KF-AMP-AML	ND	8.3
KF-AMP-OT	ND	8.3
KF-OT-NA-AMP	ND	8.3

AK- Amikacin; KF- Cephalothin; F- Nitrofurantoin; OT- Oxytetracycline; AMP-Ampicillin; AML- Amoxycillin; NA-Nalidixic Acid; CN-Gentamicin; ND- No resistance

VIRULENCE GENES IN *E. COLI* ISOLATED FROM THE LOSKOP CANAL AND THE SKEERPOORT RIVER

In the Loskop canal 15.7% of isolates were positive for at least one virulence gene. Additionally 10.5% of isolates were positive for *stx1*/*stx2* and *eae* and 5.2% for *stx1*/*stx2* (Table 8). In the Skeerpoort river, 41.6% of isolates were positive for at least one virulence gene. Additionally 25% of isolates were positive for *stx1*/*stx2* and *eae* (Table 8). Furthermore 8.3% of isolates in the Skeerpoort river were positive for only *stx1/stx2* and *eae* respectively. All isolates possessing virulence genes in the Loskop canal showed antibiotic resistance. In the Skeerpoort river, 80% of isolates possessing virulence genes showed antibiotic resistance.

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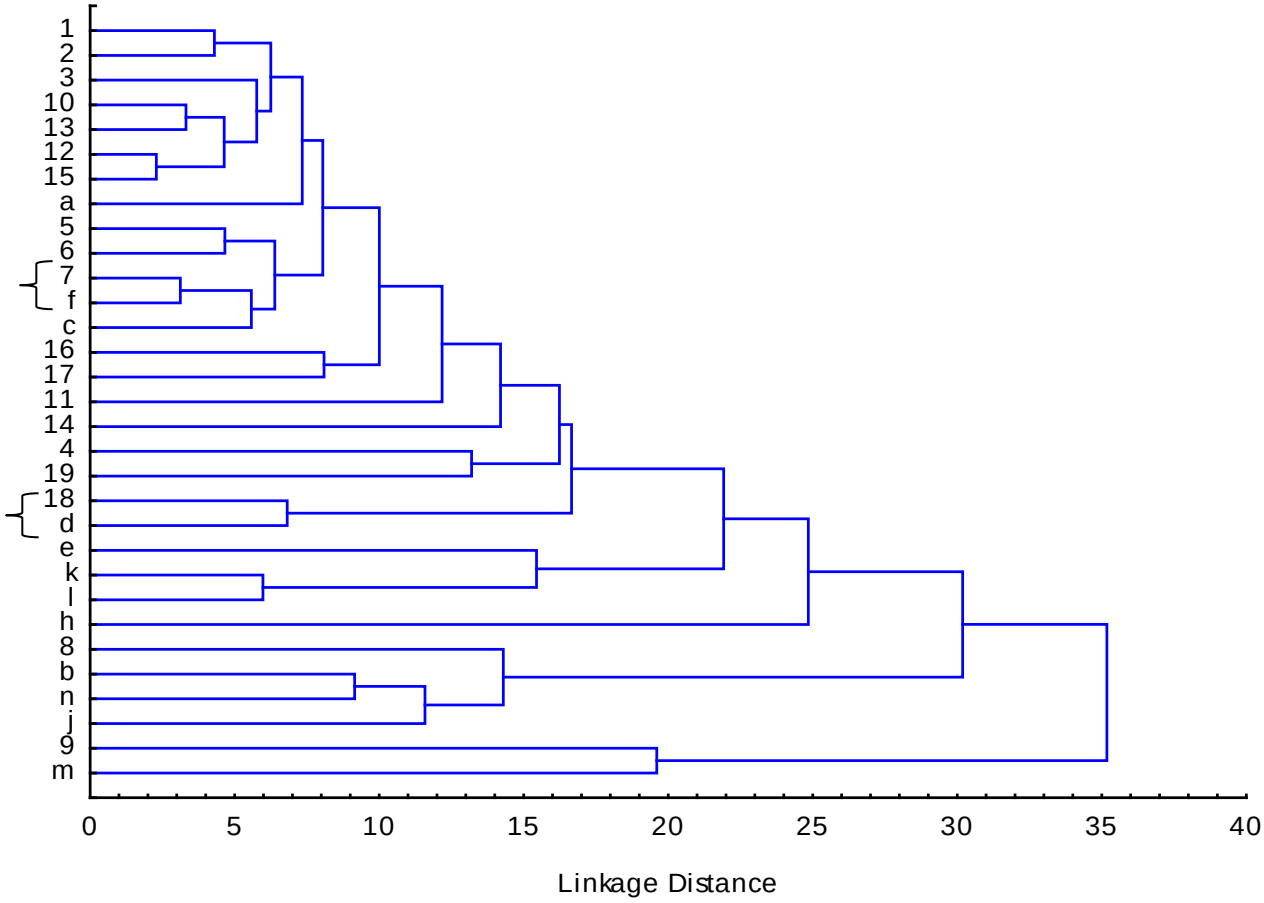

Figure 14: Dendrogram showing cluster formation for *E. coli* collected over 10 months from the Loskop canal and the Skeerpoort river. Numbers represent isolates from the Loskop canal while alphabetical letters represent isolates from the Skeerpoort river. Brackets enclose closely related isolates from the two sites

Table 8: Prevalence of virulence genes in *E. coli* isolated over 10 months from the Loskop canal, the Skeerpoort river and lettuce irrigated with water from the Skeerpoort river

Source	% occurrence of virulence genes		
	Stx 1/*Stx2* and *eae*	*Stx 1*/*Stx 2*	*eae*
Loskop canal	10.5	5.2	ND
Skeerpoort	25.0	8.3	8.3
Lettuce	ND	20.0	ND

stx 1- Shigatoxin 1 gene *stx* 2-Shigatoxin 2 gene *eae*-Intimin gene ND-not determined

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ANTIBIOTIC RESISTANCE AND VIRULENCE GENES IN *E. COLI* ISOLATED FROM THE SKEERPOORT RIVER AND IRRIGATED LETTUCE

E. coli from the Skeerpoort river and irrigated lettuce were resistant to the same antibiotics (amoxycillin, ampicillin, cephalothin and oxytetracycline) except nalidixic acid, norfloxacin and neomycin to which isolates in the Skeerpoort river were additionally resistant to (Table 9). There were 83.3% and 90% of isolates resistant to at least one antibiotic in the Skeerpoort river and lettuce respectively. Highest resistance to single antibiotics was noted to both ampicillin and cephalothin in *E. coli* from both sources (Table 9).

Table 9: Prevalence of antibiotic resistant *E. coli* collected over 10 months from the Skeerpoort river and lettuce irrigated with water from the Skeerpoort river

	% of Resistant Isolates	
Antibiotic	Skeerpoort river (n=12)	Lettuce (n=10)
Amikacin, 30µg	ND	ND
Gentamicin, 10µg	ND	ND
Chloramphenicol, 30µg	ND	ND
Nalidixic acid, 30µg	16.7	ND
Norfloxacin, 10µg	8.3	ND
Neomycin, 30µg	8.3	ND
Nitroforantoin, 300µg	ND	ND
Amoxycillin, 25µg	16.7	10.0
Ampicillin, 10µg	50.0	50.0
Cephalothin, 30µg	50.0	60.0
Oxytetracyline, 30µg	25.0	20.0

ND - No resistance

Resistance of isolates to more than one antibiotic was 50% in both the Skeerpoort river and from lettuce. However in the Skeerpoort river, 33.3% of isolates were resistant to more than 2 antibiotics. There was a significant difference ($p \leq 0.05$) in resistance to individual antibiotics among *E. coli* from the Skeerpoort river and lettuce. All isolates from lettuce were susceptible to norfloxacin, nitrofurantoin, nalidixic acid, gentamicin, chloramphenicol, amikacin and neomycin. Isolates from lettuce were more susceptible to antibiotics than those from the Skeerpoort river (Table 9). Three antibiotic resistance patterns were noted among isolates from lettuce (Table 10). More isolates from the Skeerpoort river showed resistance to multiple antibiotics (Table 10).

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Most isolates from the same sample showed closer phenotypic relatedness based on resistance to the antibiotics compared to those from different samples (Figure 15). However 40% of isolates (i and c, d, f, h, j; ii and k; iii and e; x and m) on irrigated lettuce showed close phenotypic relatedness based on antibiotics with those from irrigation water in the Skeerpoort river (Figure 15).

Table 10: Multiple resistances to antibiotics in *E. coli* collected over 10 months from the Skeerpoort river and lettuce irrigated with water from the Skeerport river

	% of isolates with indicated resistance pattern	
Pattern of antibiotic resistance	Skeerpoort river (n=12)	Lettuce (n=10)
KF-AMP	8.3	20.0
KF-OT	8.3	ND
KF-AMP-NA	8.3	ND
KF-AMP-AML	8.3	ND
KF-AMP-OT	5.3	ND
KF-OT-NA-AMP	8.3	ND
AMP-AML	ND	10.0
AMP-OT	ND	20.0

AMP, Ampicillin; KF, Cephalothin; OT, Oxytetracycline; NA, Nalidixic acid ; ND, No resistance

VIRULENCE GENES IN *E. COLI* ISOLATED FROM THE SKEERPOORT RIVER AND IRRIGATED LETTUCE

In the Skeerpoort river, 41.7% of isolates were positive for at least one virulence gene. Additionally 25% of isolates were positive for *stx1*/*stx2* and *eae* (Table 8). Furthermore 8.3% of isolates in the Skeerpoort river positive for only *stx1/stx2* and *eae* (Table 8). From Lettuce, 20% of isolates were positive for *stx1*/*stx2* genes (Table 8).

In the Skeerpoort river, 80% of isolates possessing virulence genes isolates showed antibiotic resistance. From lettuce all isolates possessing virulence genes showed antibiotic resistance.

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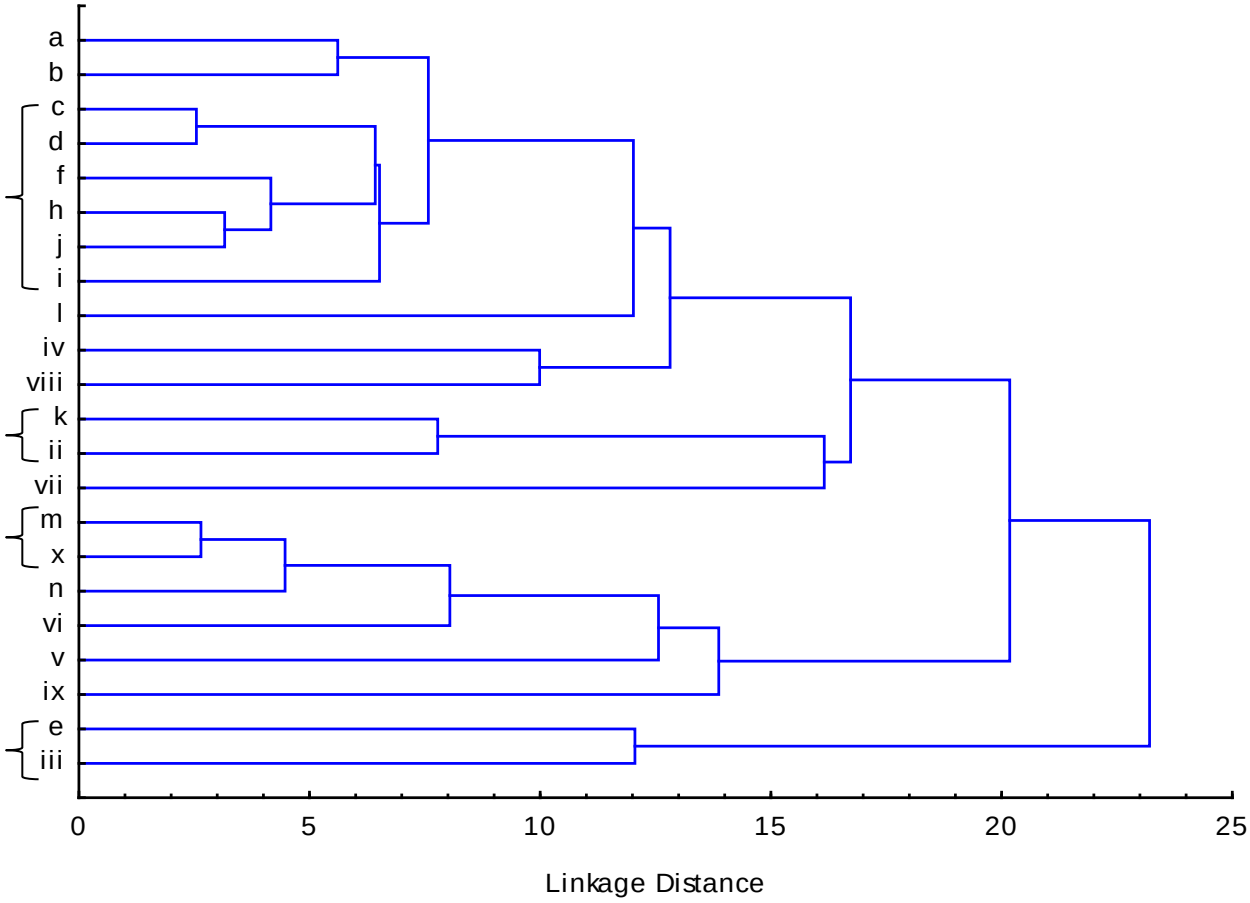

Figure 15: Dendrogram showing cluster formation for *E. coli* collected over 10 months from the Skeerpoort river and lettuce irrigated with water from the Skeerpoort river. Alphabetical letters represent isolates from the Skeerpoort river while roman numerals represent isolates from lettuce. The isolate 'i' is a roman numeral. Brackets enclose closely related isolates from the two samples

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3.3.5 DISCUSSION

High resistance of *E. coli* isolated from the Loskop canal and the Skeerpoort river to cephalothin and ampicillin may suggest extended exposure to these antibiotics probably resulting from common use within areas surrounding the two sites. Additionally resistance of *E. coli* from lettuce and irrigation water from the Skeerpoort river to similar antibiotics may have been indicative of irrigation water as a source of bacterial contamination. Extended exposure of *E. coli* to antibiotics has been noted to increase resistance because resistance genes can be mobilized through horizontal genetic transfer that occurs in mobile genetic elements such as plasmids (Da Silva and Medonça, 2012). Beta-lactam antibiotics, to which ampicillin and cephalothin belong, have low toxicity, a factor that has resulted in over use of these drugs within the medical community (Olaniran *et al*., 2009). High resistance to ampicillin and cephalothin was also noted in *E. coli* isolated from the Umgeni and Palmiet rivers (Olaniran *et al*., 2009).

Higher multiple resistances to antibiotics within *E. coli* isolated from the Skeerpoort river than the Loskop canal may be linked to existence of a large pool of antibiotic resistance bacteria. Rivers flowing through urban areas may be exposed to higher levels of pollution compared to rural areas through domestic and industrial waste (Walters *et al*., 2011) increasing the risk of contamination with antibiotic resistant bacterial pathogens. Olaniran *et al*., (2009) suggested that contamination of surface water sources with pollutants might be linked to increased prevalence of antibiotic resistant pathogens. Therefore irrigation water sources may serve as reservoirs of antibiotic resistant pathogens posing a food safety and public health threat in case new pathogens emerge. Multiple resistances to antibiotics were noted in *E. coli* isolated from rivers close to urban/densely populated areas (Olaniran *et al*., 2009; Da Silva *et al*., 2011).

The close phenotypic (antibiotic resistance) relatedness shown by some *E. coli* from the Loskop canal and the Skeerpoort river may have resulted from extended exposure to similar antibiotics. *E. coli* from the Loskop canal and the Skeerpoort river may have been exposed to similar antibiotics in spite of the geographical difference. This may have been due to spontaneous events of contamination resulting from point and non-point pollution possibly influenced by surrounding land use practices. Therefore in spite of different geographical location, the Loskop canal and Skeerpoort river may have been exposed to similar sources of contamination.

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On the other hand phenotypic relatedness of *E. coli* from lettuce and irrigation water from the Skeerpoort river suggested the river may have been a source of bacterial contamination on irrigated lettuce.

The presence of genes associated with EHEC infections within *E. coli* from the Loskop canal, Skeerpoort river and lettuce irrigated with water from the Skeerpoort river suggests the ability to cause Shigatoxin related human infections. Additionally resistance to antibiotics noted within these virulent strains heightens the risk of illness as well as resulting health complications among diseased individuals due to the reduced effectiveness of antimicrobial therapy. Therefore irrigation water containing virulent and antibiotic resistant *E. coli* is unsuitable for irrigating fresh produce because such produce normally undergoes minimal processing that does not eliminate all pathogens (Pachepsky *et al*., 2011). The majority of foodborne outbreaks associated with *E. coli* in fresh produce have been linked to EHEC (Weiss *et al*., 2011). *E. coli* isolates coding for *stx1*, *stx2* and *eae* genes were noted as resistant to ampicillin and streptomycin (Da Silva and Medonça, 2012). Studies have reported on high resistance to antibiotics in Shigatoxin producing *E. coli* isolated from animal waste water (Schroeder *et al*., 2002; Da Silva and Medonça, 2012).

3.3.6 CONCLUSION

Results from this study show that surface irrigation water sources in South Africa can be reservoirs of *E. coli* possessing virulence and antibiotic resistant genes. Additionally irrigation water with poor bacteriological quality may contaminate irrigated produce presenting a risk to both food safety and public health. Therefore continuous and consistent monitoring of the bacteriological quality of irrigation water sources is essential in order to assess levels of pathogenic bacterial contamination as well as determine potential sources of contamination for remediation measures. There is a need to determine the link between antibiotic resistance and virulence in bacterial pathogens isolated from environmental sources. Such information would help determine inherent and/or environmental factors that may influence acquisition of antibiotic resistant and virulence genes within bacterial pathogens providing better information for assessing risks posed to food safety and public health.

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CHAPTER 4: GENERAL DISCUSSION

4.1 CRITIQUE OF METHODOLOGY

This study set out to assess the bacteriological quality of irrigation water from the Loskop canal and the Skeerpoort river based on difference in geographical location. Additionally the study aimed to determine whether irrigation water from the Skeerpoort river was a source of bacterial pathogens on irrigated lettuce grown under field conditions.

Collection of samples at each irrigation water source was done at points in close proximity to each other throughout the 10 month study something which may have biased results depicting levels of bacterial contamination in the Loskop canal and the Skeerpoort river. This is because both irrigation water sources flow over large geographical areas exposing them to increased risk of bacterial contamination from human and environmental sources due to point and non-point pollution. Demarcation and studying of bacterial contamination over specified points along the Loskop canal and the Skeerpoort river over the same 10 month period may have provided a better sample space for use in representing the wider area. Da-Silva *et al*., 2011 and Richney and Coyne (2009) both determined the bacteriological quality of river sources by sampling at different sites along the respective water sources.

The use of genotypic methods such as molecular based methods involving PCR may have helped accurately screen for bacterial pathogens during the study hence saving time and other resources. This is because many of the culture based methods although internationally validated provided false positive results during identification and characterization of common bacterial pathogens such as *Salmonella* spp, *E. coli* and *L. monocytogenes*. Therefore genotypic methods had to be used to confirm some of the bacterial isolates. In relation to giving false positive results, identification of *Salmonella* spp from the two irrigation water sources and lettuce using cultural methods led to numerous misidentifications prior to serotyping. Isolates identified as *Salmonella* spp on 5 of the 10 months in the Loskop canal were serotyped as; *Pseudomonas putida, Vibrio metschinikovi, Enterobacter cloacae* and *Citrobacter freundii*. In the Skeerpoort river isolates were serotyped as; *E. aerogenes, Klebsiella pneumoniae, Pseudomonas flourescens, Vibrio damsela, Aeromonas hydrophila, Bacillus coagulans* and *Citrobacter freundii* on 7 of the 10 months during the study. On lettuce isolates were serotyped as *P. putida* and *C. freundii* during 2 of the 10 months of the study.

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In bid to use antibiotic resistance as a means of determining inter-isolate relatedness among environmental *E. coli*, using a small number of isolates in the study was noted to reduce reliability of results (Whitlock *et al*., 2002). When using antibiotic resistance as an MST method, using a large number of isolates is recommended (Scott *et al*., 2002). Additionally coupling antibiotic resistance with genotypic methods may have provided more conclusive information relating to relatedness of *E. coli*. This is because genetic compositional measurements are more resolute and strain specific compared to phenotypic measurements which may be acquired through horizontal genetic transfer within the environment from related and unrelated species.

Although the real time PCR screened for some virulence genes in the *E. coli*, it could not differentiate presence of *stx1* and *stx2* hindering the conclusive validation of individual shigatoxins. *Stx2* has been noted as having higher toxicity than *stx1* and is also more associated with food related outbreaks (Welch, 2006). Therefore confirmation of individual toxins is crucial in assessing the food safety risk posed by virulent strains of *E. coli*. Additionally the use of a method enabling characterization of more virulence genes (other than *stx1*, *stx2* and *eae*) may have provided better representation of potential pathogenicity of isolated *E. coli*. This is because virulence genes within bacterial pathogens may pose a threat to food safety. The outbreak strain *E. coli* O104: H4 was found to have a number of previously uncharacterized virulence factors that were suggested to have made it highly pathogenic (Rohde *et al*., 2011). Therefore screening for other virulence factors within *E. coli* not necessarily associated with EHEC infections may be necessary to assess the food safety and health risks posed by pathogenic *E. coli.*

4.2 INFLUENCES OF BACTERIAL CONTAMINATION IN THE LOSKOP CANAL, SKEERPOORT RIVER AND LETTUCE IRRIGATED WITH WATER FROM THE SKEERPOORT RIVER

Irrigation water collected from the Loskop canal and the Skeerpoort river is contaminated with a wide range of potentially pathogenic bacterial species among which are *E. coli* and *K. pneumonia* that have been linked to causing disease in humans. This study set out to compare pathogenic bacterial contamination based on geographical location (provinces) and possible land use practices (rural and urban) in the Loskop canal and Skeerpoort river respectively, however results showed lack of a clear-cut difference in these levels at both sites. Such an observation suggests influence of bacterial contamination from uncharacterized sources.

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For example uncharacterized sporadic pollution events in water sources such as spillages may cause more contamination which may not necessarily result from geographical location and land use factors. Additionally sporadic pollution events may be promoted by seasonal weather influences (rainfall precipitation) that was specifically noted in this study to increase concurrently with levels of faecal indicators (faecal coliforms) and incidence of pathogens (*E. coli*) in both water sources. Rainfall can carry bacterial contamination over large geographical areas therefore protection of irrigation water sources from runoff may help limit pollution of irrigation water sources. Seasonal temperature was also noted to influence prevalence of specific pathogens such as *E. coli* (winter) and *Bacillus* spp (summer). Information relating to seasonal bacterial pathogen prevalence may assist stakeholders such as farmers, food safety experts and policy makers better assess risks associated with irrigation water and fresh produce in a bid to maintain food safety and public health as well as maintain a vibrant, profitable fresh produce industry in South Africa.

The low effectiveness of bacterial indicators to reliably reflect pathogenic bacterial contamination compared to non-selective media suggested that it may not provide reliable results when used to assess environmental contamination. This is because such methods may underestimate pathogenic bacterial contamination since some pathogens when subjected to unfavourable conditions such as those within environmental settings revert to the 'viable but non-culturable (VBNC)' state making them unculturable on media (Dreux *et al*., 2007). Failure of VBNC cells to grow on selective agar is due to the net increase of hydrogen peroxide resulting from metabolic activities of the cells coupled with levels that may be present within selective agar ultimately leading to quantities toxic to cells (Oliver, 2005). Additionally the high nutrient content of selective media maybe toxic to bacterial cells in the VBNC state (Dreux *et al*., 2007). Therefore during investigative studies aimed at assessing the bacterial quality of environmental sources, screening for specific pathogens such as *E. coli* using non-selective media may provide better information relating to prevalence of these bacterial pathogens.

The isolation of similar pathogens in irrigation water from the Skeerpoort river and irrigated lettuce suggested that irrigation water has the potential to contaminate fresh produce compromising the bacteriological quality of fresh produce and food safety of consumers. Additionally it suggests ability of pathogenic bacteria to survive pre-harvest environmental conditions.

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This may increase the risk of disease in consumers if all pathogens are not eliminated during post-harvest processing as could possibly occur with minimally processed fresh produce such as lettuce. The wider range of bacterial species isolated from irrigated lettuce compared to irrigation water from the Skeerpoort river suggested different sources of bacterial contamination on produce. Other sources of bacterial contamination may have included; soil, wild animals and human handlers. Presence of different bacterial species may influence proliferation of bacterial pathogens through formation of biofilms and in the process compromise food safety and public health.

High prevalence of antibiotic resistance among *E. coli* from the Loskop canal, the Skeerpoort river and lettuce irrigated with water from the Skeerpoort river especially to beta-lactamase antibiotics such as cephalothin and amipicillin suggested distribution of antibiotic resistant bacteria within the two water sources. Transfer of antibiotic resistant genes has been noted to easily occur between similar species repeatedly exposed to antibiotics (Da Silva and Medonça, 2012). Therefore these results suggest that there might have been repeated exposure of *E. coli* within the Loskop canal and the Skeerpoort river to antibiotics that may have resulted from pollution caused by domestic and industrial sources. Antibiotic resistance genes can easily be transferred on mobile genetic elements located on plasmids of related and unrelated species thereby increasing the risk of commensal bacteria within these water sources developing antibiotic resistance. This may lead to emergence of new pathogens which could be transferred onto irrigated fresh produce compromising food safety and public health.

The presence of virulence genes (*Stx1*, *Stx2* and *eae*) associated with Enterohaemorrhagic *E. coli* (EHEC) in *E. coli* from the two irrigation water sources and on lettuce suggested ability to cause EHEC related human infections. Foodborne outbreaks in fresh produce associated with Shigatoxin producing *E. coli* have been reported (Rohde *et al*., 2011; Rasko *et al*., 2011). Therefore *E. coli* in possession of virulence genes from the Loskop canal, the Skeerpoort river and on irrigated lettuce may have the ability to cause foodborne outbreaks. Although the link between antibiotic resistance and virulence requires more investigation (Da Silva and Medonca, 2012), presence of both antibiotic resistant and potentially virulent *E. coli* from both irrigation water sources and on lettuce heightens the risk of illness posed by these pathogens. This makes water from the two irrigation water sites unsuitable for irrigating fresh produce because minimal processing does not eliminate all pathogens hence posing risk of illness to the final consumer.

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The fact that both antibiotic resistant and virulence genes may be transferred on plasmids in bacterial pathogens heightens the risk of new lethal pathogens emerging as a result of increased exposure to high level of antibiotics (antibiotic pressure) as well as unfavourable environmental conditions. The acquisition of these potentially lethal properties may result from the need of bacterial pathogens to acclimatize to new environments.

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CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

Determining prevalence of specific bacterial species rather than use of bacterial indicators may provide a better means of assessing bacteriological quality of irrigation water sources and contamination on irrigated produce. The Loskop canal and the Skeerpoort river harboured antibiotic and virulent *E. coli* with contamination possibly linked to surrounding land use practices thereby presenting a food safety and health risk to the surrounding population. Antibiotic resistance among *E. coli* suggests existence of an antibiotic resistance and virulence gene pool within these water sources which may facilitate exchange of mobile genetic elements such as plasmids among related and unrelated species. This may lead to emergence of antibiotic resistant pathogens which could cause foodborne outbreaks. Fresh produce such as lettuce is susceptible to pathogenic bacterial contamination from environmental sources such as irrigation water which increases risk of disease to the final consumer especially if pathogens survive minimal post-harvest processing.

Continuous monitoring and testing of surface water sources used for irrigation is recommended regardless of geographical location since studies have shown that both urban and rural irrigation water sources can be sources of bacterial pathogens. This may help determine suitability of irrigation water for fresh produce production helping to limit contamination. Additionally this may assist in determining sources of bacterial contamination through microbial source tracking techniques helping in remediation measures.

In order to limit pre-harvest pathogenic bacterial contamination of fresh produce by irrigation water, studies should be initiated to investigate the die off period of pathogens after irrigation with untreated water. This may help determine the satisfactory time at which to irrigate crops with untreated irrigation water such that harvested produce is free from bacterial pathogens. This alternative may provide a more cost effective means for farmers limiting pre-harvest bacterial contamination since water treatment may prove costly.

The use of barriers, protected wells and dams provides a means of protecting irrigation water from environmental sources of bacterial contamination such as runoff, wild and domestic animals. This may limit bacterial contamination in water sources reducing transfer onto irrigated produce.

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The mode of irrigation influences pathogenic bacterial contamination on irrigated produce. Surface irrigation has been noted to limit contamination of aerial parts of plants compared to sprinkler/spray irrigation. Therefore using surface irrigation may limit pre-harvest contamination.

More environmental based studies relating to factors (inherent to the plant and pathogen) favouring pathogenic bacterial proliferation should be carried out to help better understand the mechanism which lead to proliferation of bacterial pathogens on fresh produce.

This would help provide reliable information for use in implementing measures for limiting pre-harvest proliferation of bacterial pathogens thereby reducing risk of foodborne illness to consumers.

The use of genotypic finger-printing methods may provide a better means for determining relatedness among isolates of the same species. Coupling antibiotic resistance with genetic fingerprinting would provide more accurate information on relatedness of bacterial pathogens and possible sources of contamination. Additionally reliably determining sources of faecal contamination requires formation of a reference data base of *E. coli* or faecal coliforms collected from possible sources of contamination within the environment such as domestic animals, humans and sewage. Using such as database would provide a better comparison and hence more accurate information relating to sources of faecal contamination within irrigation water sources and on irrigated produce.

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