INVESTIGATION INTO THE LINK BETWEEN WATER QUALITY AND MICROBIOLOGICAL SAFETY OF SELECTED FRUIT AND VEGETABLES FROM FARMING TO PROCESSING STAGES (VOLUME 2)

Report to the
WATER RESEARCH COMMISSION
and
DEPARTMENT OF AGRICULTURE, FORESTRY AND FISHERIES

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EXECUTIVE SUMMARY

Background and motivation

An increase in the consumption of fresh and minimally processed fruits and vegetables globally (Ayers and Westcot, 1985; Beuchat, 2002) has resulted in one of the most important health challenges in terms of foodborne diseases. In the United States of America (USA) these dietary changes resulted in doubling of produce-associated disease outbreaks per year from 1973-1992 (USA Food and Drug Administration, 1998). However, over the past 10-15 years the number of foodborne disease outbreaks has declined. The number of CDC outbreak reports varied between 1243 to 1417 during 2000-2002 in comparison to only 800 to 850 outbreaks reported from 2009-2013 (http://www.foodsafetynews.com/2015/06/the-prevalence-of-foodborne-illness; accessed 12 July 2015). Specific examples include three outbreaks in 2004 in the USA and Canada due to Salmonella, affecting 561 people who consumed fresh tomatoes [Centres for Disease Control and Prevention (CDC), 2005]. In 2006 another outbreak was associated with the consumption of tomatoes, with 183 cases being reported in 21 states (CDC, 2006a). Subsequently, a *Salmonella* outbreak, occurring from April to August 2008 in the USA, was linked to the consumption of raw tomatoes, jalapeno and cilantro. This multi-state outbreak affected 1438 people with 282 people needing hospitalisation and possibly contributing to the deaths of two people (CDC 2008a). In 2008 a second outbreak was associated with cantaloupe. This affected 51 people in 16 states, with 16 people hospitalised (CDC 2008b).

Major disease outbreaks have also been associated with other pathogens such as *Escherichia coli* O157:H7, which affected 183 people in the USA in September 2006. Of these, 95 people were hospitalised, 29 people had haemolytic uremic syndrome and one person died (CDC 2006b). This outbreak was linked to the consumption of fresh bagged spinach. Additionally, a more recent multistate outbreak of *E. coli* O157:H7 in 2011 in 10 states of the United States caused 60 individuals to become ill (http://www.cdc.gov/ecoli/2011/ecoliO157/romainelettuce/120711/index.html; accessed 2015 June 25). A foodborne disease outbreak in the European Union in May 2011 was caused by *E. coli* O104:H4, a more virulent verocytotoxin-producing strain than *E. coli* O157:H7. The outbreak was eventually linked to a German sprout producer after first implicating Spanish cucumbers and tomatoes. The resultant €225 million losses per week of Spanish vegetable producers highlighted the economic impact of these outbreaks and the importance of accurate diagnostic test methods (http:// www.bbc.co.uk/news/world-europe-13683270; accessed 2015 June 25). A Listeriosis outbreak during 2011 was reported to be due to consumption of contaminated cantaloupes that left 146 people ill and caused 30 deaths in 28 states across the United States (http://www.foodsafetynews.com/2011/10/Cantaloupe-listeria-outbreak-84-sick-15-dead; accessed 2015 June 25).

According to the World Health Organisation about one third of 51 million deaths world-wide result from infectious and parasitic diseases. It has been estimated that worldwide 50 000 people die daily as a result of water-related diseases. Foodborne diseases due to food

contamination may occur at any point along the food chain, from the farm through food processing to the end user. However, the majority of foodborne outbreaks have been traced to contamination on the farm and the use of contaminated water (Westcot, 1997; Beuchat, 2002). Several studies have investigated or reviewed the impact of contaminated irrigation water on the spread of foodborne diseases (Ayers and Westcot, 1985; FDA, 1998; Venter, 2001).

Fresh produce is important from a national food supply and consumer health point of view while fresh fruit exports constitute a major part of the economy representing the third biggest earner of foreign exchange. South Africa has over the past 100 years built an international reputation as a major competitor in the export markets, delivering top quality and safe produce. South African farmers have further implemented measures to ensure compliance with new stringent good agricultural practices and food safety standards, allowing the country to remain a leading player in international food trade.

Unprecedented challenges facing the quality and quantity of our national water supplies have resulted in a new risk for farmers reliant on irrigation of cultivated crops (Gemmel and Schmidt, 2012; Chigor *et al*., 2013). Studies solicited by the Water Research Commission (WRC) entitled: "Quantitative Investigation into the Link between Irrigation Water Quality and Food Safety" (WRC Report Number 1773/1-4/12) and "Health risk assessment in connection with the use of microbiologically contaminated source water for irrigation" (WRC Report Number 1226/1/04) have shown that the national water supplies are contaminated with pathogens and may pose a potential risk for the fresh fruit export industry and national safe food supplies. It was argued that a project that focuses on the risks in the supply chain will support and strengthen the findings in WRC Report Number 1773/1-4/12.

Aims:

The general and specific aims of this project were:

1. To investigate the links between water quality and microbiological safety in fresh minimally processed and frozen fruit and vegetables.
2. To review the literature study of WRC Report Number 1773/1-4/12 and expand the review to include a comprehensive literature study on processing water quality and its impact on the microbiological safety of food and potential effect on human health.
3. To establish co-operation with the team of researchers involved in WRC project K5/1773/4.
4. To select appropriate sites, with reference to i) Different sources of untreated and treated water (excluding greywater) ii) Different types of microbial contamination iii) Different types of farming – Subsistence, emerging, commercial farming for local and export markets iv) Different crops and produce (specifically fruit and vegetables) v) Different pathways of contamination: water for irrigation and water used for sprays vi) Different pathways of contamination: water as transport and washing medium as part of packing and processing.

5. To propose and standardise the research methodology for the assessment of the impact of contaminated irrigation water and processing water on microbial food safety.
6. To determine the extent (types and quantities) of contamination found in irrigation and processing water at the selected sites, with reference to i) Indicators of water quality ii) Volume of water used for irrigation and processing iii) Microbial contaminants (viruses, bacteria, parasites) iv) Physical parameters (pH, COD, EC, turbidity, temperature, etc.) v) Influence of personal on-site hygiene facilities vi) Influence of external quality control on food safety management at the farming and processing stages of production.
7. To investigate the impact of environmental parameters on the growth kinetics of contaminants, with reference to i) fate and survival ii) Decay rates iii) Surrogate organisms iv) Different irrigation and processing technologies.
8. To study the extent of (types and quantities) contamination found on the irrigated raw produce (fruit and vegetables) up to harvest at selected sites, with reference to i) Microbial contaminants (viruses, bacteria, parasites) ii) Microbial indicators/index of contamination/risk iii) Method of irrigation, agri-chemical spray water application, etc. iv) Investigate the potential link and/or association between contamination on raw produce and water applied (molecular epidemiology using a reference organism).
9. To study the extent (types and quantities) of contamination found on the raw produce (fruit and vegetables) after harvest, with reference to i) Microbial contaminants (viruses, bacteria, parasites) ii) Microbial indicators/index of contamination/risk iii) Additional contamination through handling, washing, storage, packaging, pre-freezing and minimal processing, etc. iv) Impact of biofilms in processing on product contamination.
10. To make interim and final recommendations for further research, with reference to: Further validation of results and Effective treatment options.

Research Findings

In this volume the results for aims 8-10 (see page iv-v) including the potential link between the microbiological quality of irrigation water and fresh produce quality and safety are reported.

Aims 8 and 9: Microbial contamination found on the raw produce (fruit and vegetables) up to harvest (including at harvest) as well as after harvest

Since the presence of pathogens on different crops has been reported and its prevalence has been linked to specific surface characteristics (hairy, smooth, etc.), a broad range of fresh produce (including minimally processed fruit and vegetables) were sampled from farms, processing factories, fresh produce markets, street vendors and retailers. Current microbiological specification guidelines by the Department of Health (DoH) for ready-to-eat fresh fruit and vegetables require coliform counts of less than 200/g, yeast and mould counts of < 100 000 (log 5)/g, *Escherichia coli* 0/g and *Salmonella* at 0/25 g. The microbial levels on crop surfaces were found to be lower further down the supply chain as was evident from

results obtained for specific crops sampled “up to” and “at” harvest on the farm as well as “after” harvest from processing factories, the fresh produce market and retailers.
Staphylococcus aureus, Listeria spp., *Salmonella* spp., commensal *E. coli* and diarrhoeagenic *E. coli*, as well as viruses [Noro Virus (NoV) GI, NoV GII, and hepatitis A virus (HAV)] were detected at certain stages along the vegetable and fruit supply chain, but not from the beginning to the end of the chain. It was evident that the fresh produce with the highest microbial contamination were leafy vegetables (lettuce and spinach) and herbs (basil and parsley).

Generally the microorganism (bacteria) levels present on vegetable products were higher when compared to fruit samples. This is an important finding since it confirmed previous reports that fruit in general is by its very nature and production system a lower risk product when compared to vegetables, which are mostly grown in closer proximity to the ground. Basil is often consumed raw and was found to harbour coliform, yeast and mould counts higher than the DoH specifications. *Salmonella* Typhimurium was present in some of the packhouse basil samples tested, which pose a potential health risk.

NoV GII was detected in the parsley samples from the field and from the packhouse, which poses a potential health risk, since parsley is often consumed raw. Swine NoV GII.18 was detected in strawberries and was the first report of this virus in South Africa. Subsequently, the possibility of zoonotic NoV infection was raised. Interestingly, swine NoV GII.18 was detected in the strawberries only when overhead irrigation was used to cool down the strawberries when outside temperatures were high. The fresh produce with the highest microbial contamination was leafy vegetables (lettuce and spinach).

Aim 10: Recommendations for further research
Refer to Chapter 4, section 4.2 of this volume.

General aim: Links between microbial contamination on raw produce at harvest, stages of processing after harvest and water applied
Over the 6 year period at the 17 sites tested, we found a clear link between the microbiological quality of the irrigation water and produce (onions and lettuce) at only two of the sites tested using phenotypic (antimicrobial) and genotypic (virulence gene prevalence, DNA fingerprinting) analysis of *E. coli* isolates (see section 3.2.8 and 3.2.10). The low prevalence of human pathogenic bacteria and the fact that it was not isolated along the entire supply chain of a specific crop, was a major limiting factor to using microbial source tracking. This approach is used to determine if irrigation water can be the source of the pathogens isolated from fresh produce. It is also an important factor to be taken into account when drawing conclusions about the absence of a pathogen in a specific crop (Lopez-Galvez *et al.* 2014).

Details of the case studies are summarised below:

Irrigation water and parsley:
Norovirus GII was not detected in the irrigation water at the time of sampling, but it was detected on parsley sampled in the field and in the packhouse. Irrigation water could thus not be shown to be the source of contamination at this sampling site. Since the irrigation crop link could not be confirmed in this study no definite assumptions could be made regarding the source of contamination at the time of sampling. The presence of NoV GII on the parsley could pose a potential health risk, since parsley is often consumed raw. It has been reported that sixty percent of foodborne disease outbreaks in the United States linked to leafy green vegetable consumption between 1973 and 2006 was caused by Norovirus (http://www.sciencedaily.com/releases/ 2008/03/080317164356; accessed 12 July 2015).

Irrigation water and cabbage:
None of the viruses tested for (NoV GI, NoV GII and HAV) were detected in the river and borehole samples from a small-scale farm in Gauteng. Similarly no viruses could be detected at the point of sale (street vendors selling cabbages). Although the river water sample from a KZN farm tested positive for HAVgenotype V, no viruses (NoV GI, NoV GII, and HAV) were detected in any of the cabbage samples collected from the irrigated farm. Since no viruses were detected on the cabbages, a direct link between contaminated irrigation water and associated fresh produce could not be established. WRC report no. 1773/4/12 reported the presence of NoVGII.1 on one cabbage sample irrigated with water from the Berg River in the Western Cape. However, no correlation between the occurrence of viruses in irrigation water samples and linked fresh produce samples could be made in that report. This report was thus in agreement with previous findings.

Irrigation water and tomatoes :
Although water used to irrigate tomatoes on a farm in Limpopo was found to be contaminated with human enteric viruses including HAV, NoV GI and NoV GII, indicating potential human faecal pollution, none of the viruses were isolated from the tomatoes in this study. This could be due to the fact that the tomatoes are drip irrigated and do not come into direct contact with the irrigation water. In WRC report no. 1773/4/12 NoV GII.4 and NoV GII.2 were detected in irrigation water samples. NoV GII.9 and HAV 1B (indicating human faecal contamination) were also detected on tomatoes and lettuce from a street vendor in Marabastad.

Irrigation water and onions:
In the case of a highly contaminated river, a positive correlation between the irrigation water source and the crop irrigated, using overhead irrigation (irrigation pivot point) could be made. *E. coli* was isolated from 78.4% of the irrigation water samples (including river, dam and irrigation pivot point samples) and 8.3% onion samples after selective enrichment and streaking onto selective media. The *E. coli* isolates were subjected to antibiotic resistance, virulence gene and Eric-PCR analysis in order to establish whether the strains from the irrigation water sources and onions were similar on a phenotypic and genetic level respectively.

Of the 45 *E. coli* isolates, 15.5% were resistant to one antibiotic and 42.2% were resistant to multiple antibiotics. Cluster analysis based on antibiotic resistance profiles for *E. coli* isolates clearly grouped some of the river, dam, irrigation pivot point and onion samples at similarity values ranging between 67% and 69% which showed a positive link between the irrigation water sources and the onion isolates on a phenotypic level.

The individual virulence genes (*eae, stx1,* and *stx2*) presence varied between 2.2% to 6.6% of the *E. coli* isolates. Eric PCR analysis clearly grouped some of the river, dam, irrigation pivot point and onion samples at similarity values ranging between 80% and 92% which showed a positive link between the irrigation water sources and the onion isolates on a genotypic level. Specific examples showing close genetic relatedness included 1) one of the onion *E. coli* isolates was 92% similar to a water *E. coli* isolate from the irrigation pivot point; 2) another river isolate and a dam water isolate were 90% similar and both harboured the *stx1* gene; and 3) one river isolate sampled further upstream from the river on the farm, one isolated from the same river on the farm and one onion isolate clustered together at a remarkable similarity value of 92%. These results clearly showed a link between *E. coli* strains from the river to the dam, irrigation pivot point and the onions. However, it is important to note that the *E. coli* was not further detected in the same onion field after harvest following drying in the middens in the field and at the fresh produce market.

Irrigation water and lettuce:
The potential link between contaminated river water and irrigated lettuce was determined using antibiotic resistance profiles of *E. coli* isolated from these two sources. Eighty three percent (83%) of *E. coli* isolates from the river were resistant to at least one antibiotic, while 90% of isolates from lettuce were resistant to at least one antibiotic. The highest resistance to single antibiotics in the two samples was to cephalothin and ampicillin. High resistance (33.3%) was noted to multiple (more than 2) antibiotics in the river. Forty percent (40%) of *E. coli* isolates from irrigated lettuce showed close phenotypic relatedness to isolates from the river.

Genetic relatedness of *E. coli* isolates from irrigation water and lettuce was further determined using GTG PCR DNA fingerprinting. There were 33% of isolates from irrigation water having 100% genetic similarity to 60% of isolates from lettuce. Results from this study showed that irrigation water could harbour antibiotic resistant *E. coli* which can be transferred onto irrigated produce. This could potentially represent a food safety risk. The result for lettuce specifically was in agreement with the WRC Report Number 1773/1-4/12 linking studies which showed at least 30 per cent correlation between irrigation water and pathogen presence on the product. However, this correlation was determined for *E. coli* isolates from irrigation water and for harvest-lettuce only and not for lettuce further down the supply chain, i.e. after harvest, at the market or the retailer.

Irrigation water and basil:
Although the irrigation water quality was acceptable according to DWAF (1996) guidelines, the microbiological quality of basil was unacceptable since coliform, yeast and mould counts for basil were higher than the DoH (2000) specifications for ready-to-eat fruit and vegetables. Furthermore, *Salmonella* Typhimurium was present in some of the packhouse basil samples and could pose a health risk, since basil is often consumed raw. The pathogen was however not detected in any of the packaged ready-to-eat basil samples from the retailers.

Irrigation water and strawberries:
Genotypic characterisation of viruses isolated from irrigation water and strawberries showed the presence of Human Norovirus GII.7 in irrigation water samples and swine NoV GII.18 in the strawberries. Although a definite link between the viruses detected in the irrigation water and those on the strawberries could not be established, a more concerning problem of the possible human-swine NoV variants was brought to light.

Irrigation water and peaches:
Phenotypic (antibiotic resistance) characterisation of *E. coli* isolates, one each from peaches collected in the orchard, river and micro irrigation pipe samples, showed that the strains from the irrigation water and peaches were sensitive to all the antibiotics (14 in total) tested. Genotypic characterisation showed that both the *stx1* and *stx2* virulence genes were present in the single *E. coli* isolate from the river. Linking studies based on genotypic and phenotypic characterisation could not be done due to the limited number of *E. coli* isolates obtained from the irrigation water (2) and peaches (1).

Irrigation water and pears:
The *E. coli* isolates from water samples from four commercial pear farms were mostly sensitive to antibiotics (14 in total). However, resistance to single antibiotics were detected for some *E. coli* isolates and included Aztreonam (ATM) or Ceftazimide (CAZ) which both belong to the Penicillin class of antibiotics or Chloramphenicol (C) which belong to the Phenicol antibiotic class. Only one isolate was resistant to two antibiotics which included ATM and CAZ. The single *E. coli* isolated from pears in the orchard of one of the farms was sensitive to all the antibiotics tested.

None of the virulence genes were detected in any of the *E. coli* isolates (18 in total) from the irrigation water on the four commercial pear farms in the single pear *E. coli* isolate. Cluster analysis of Eric-PCR DNA fingerprints showed that two *E. coli* isolates from the dam water and two isolates from the micro-irrigation pipes in the orchard on one of the farms formed a distinct cluster at an 80% similarity value. These results confirmed that the water from the storage dam was the source of the strain isolated from the micro-irrigation pipes.

General conclusions
Microbial analysis of irrigation water(five rivers, ten dams) showed that the *E. coli* levels in the irrigation water at the sites tested (fourteen in total) during this study exceeded the DWAF (1996) irrigation water guidelines of ≤ 1-1000 CFU/100 mℓ for crops to be eaten raw.

Irrigation canal water used at two of the sites tested complied with the DWAF (1996) guidelines for crops to be eaten raw. However, although the *E. coli* counts of the primary irrigation source (rivers, dams) were unacceptably high, a reduction in numbers was observed for dam water and a further reduction was observed for the water from the micro-irrigation pipes, drip-irrigation and overhead irrigation pivots. *Salmonella* was isolated from up to 22% of the water samples from a selected site. No *Escherichia coli* O157:H7 and *Listeria monocytogenes* were isolated from any of the irrigation water samples from the sites tested.

The current national guideline for fresh produce provides guidance values only for the for ready-to-eat stage of the chain (DoH, 2000). The microbial levels on crop surfaces were generally higher in the field compared to the product at the end of the supply chain. *E. coli* and diarrhoeagenic *E. coli*, as well as viruses (NoV GI, NoV GII, and HAV) were isolated from fresh produce at certain stages along the supply chain, but not from the beginning to the end of the chain. The fresh produce with the highest microbial contamination was leafy vegetables (lettuce and spinach). Interestingly, swine NoV GII.18 was detected in the strawberries. This was the first report of swine NoVs in South Africa which raised the question as to the possibility of zoonotic NoV infection.

Generally the percentage of pathogens present on vegetable products was higher when compared to fruit samples. This is an important finding since it confirms the previous reports that fruit in general is by its very nature and production system a lower risk product than vegetables. This is mainly due to production practices where the crop is grown in closer proximity to the ground and the potential to be in direct contact with irrigation water for extended periods of time is thus greater. However, some fruit types can not be grouped under this generalised statement as was found in this and the previous WRC project (WRC Report Number 1773/1-4/12) for strawberries. Strawberries are grown close to the ground and was found to harbour several viruses.

Over the 6 year period at the 17 sites tested a clear link was found between irrigation water and produce (onions and lettuce) at only two of the sites tested using phenotypic (antimicrobial) and genotypic (virulence gene prevalence, DNA fingerprinting) analysis of *E. coli* isolates. It was concluded that the accuracy of results can be correlated with sample size and test methods used. Sample size must be adequate to obtain a statistically validated result as far as pathogen presence/absence is concerned. Incidence studies further provide a snapshot of contamination potential at a specific study site and on a specific crop at a particular time during the year. Therefore, caution should be exercised when drawing conclusions from any result based on biased sampling and sample size. In addition the test methods selected and the analysis strategy further complicates the interpretation of results.

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CHAPTER 1

1. Introduction

1.1 Background, rationale and aims of research

An increase in the consumption of fresh and minimally processed fruits and vegetables globally (Ayers and Westcot, 1985; Beuchat, 2002), has resulted in one of the most important health challenges in terms of foodborne diseases. In the United States of America (USA) these dietary changes resulted in doubling of produce-associated disease outbreaks per year from 1973-1992 (USA Food and Drug Administration, 1998). However, over the past 10-15 years the number of foodborne disease outbreaks has declined. The number of CDC outbreak reports varied between 1243 to 1417 during 2000-2002 in comparison to only 800 to 850 outbreaks reported from 2009-2013 (http://www.foodsafetynews.com/2015/06/the-prevalence-of-foodborne-illness; accessed 12 July 2015). Specific examples include three outbreaks in 2004 in the USA and Canada due to Salmonella, affecting 561 people who consumed fresh tomatoes [Centres for Disease Control and Prevention (CDC), 2005]. In 2006 another outbreak was associated with the consumption of tomatoes, with 183 cases being reported in 21 states (CDC, 2006a). Subsequently, a *Salmonella* outbreak, occurring from April to August 2008 in the USA, was linked to the consumption of raw tomatoes, jalapeno and cilantro. This multi-state outbreak affected 1438 people with 282 people needing hospitalisation and possibly contributing to the deaths of two people (CDC 2008a). In 2008 a second outbreak was associated with cantaloupe. This affected 51 people in 16 states, with 16 people hospitalised (CDC 2008b).

Major disease outbreaks have also been associated with other pathogens such as *Escherichia coli* O157:H7, which affected 183 people in the USA in September 2006. Of these, 95 people were hospitalised, 29 people had haemolytic uremic syndrome and one person died (CDC 2006b). This outbreak was linked to the consumption of fresh bagged spinach. Additionally, a more recent multistate outbreak of *E. coli* O157:H7 in 2011 in 10 states of the United States caused 60 individuals to become ill (http://www.cdc.gov/ecoli/2011/ecoliO157/romainelettuce/120711/index.html; accessed 2015 June 25). A foodborne disease outbreak in the European Union in May 2011 was caused by *E. coli* O104:H4, a more virulent verocytotoxin-producing strain than *E. coli* O157:H7. The outbreak was eventually linked to a German sprout producer after first implicating Spanish cucumbers and tomatoes. The resultant €225 million losses per week of Spanish vegetable producers highlighted the economic impact of these outbreaks and the importance of accurate diagnostic test methods (http:// www.bbc.co.uk/news/world-europe-13683270; accessed 2015 June 25). A Listeriosis outbreak during 2011 was reported to be due to consumption of contaminated cantaloupes that left 146 people ill and caused 30 deaths in 28 states across the United States (http://www.foodsafetynews.com/2011/10/Cantaloupe-listeria-outbreak-84-sick-15-dead; accessed 2015 June 25).

According to the World Health Organisation about one third of 51 million deaths world-wide result from infectious and parasitic diseases. It has been estimated that worldwide 50 000 people die daily as a result of water-related diseases. Foodborne diseases due to food contamination may occur at any point along the food chain, from the farm through food processing to the end user. However, the majority of foodborne outbreaks have been traced to contamination on the farm and the use of contaminated water (Westcot, 1997; Beuchat, 2002). Several studies have investigated or reviewed the impact of contaminated irrigation water on the spread of foodborne diseases (Ayers and Westcot, 1985; FDA, 1998; Venter, 2001).

Fresh produce is important from a national food supply and consumer health point of view while fresh fruit exports constitute a major part of the economy representing the third biggest earner of foreign exchange. South Africa has over the past 100 years built an international reputation as a major competitor in the export markets, delivering top quality and safe produce. South African farmers have further implemented measures to ensure compliance with new stringent good agricultural practices and food safety standards, allowing the country to remain a leading player in international food trade.

Unprecedented challenges facing the quality and quantity of our national water supplies have resulted in a new risk for farmers reliant on irrigation of cultivated crops (Gemmel and Schmidt, 2012; Chigor *et al*., 2013). Studies solicited by the Water Research Commission (WRC) entitled: "Quantitative Investigation into the Link between Irrigation Water Quality and Food Safety" (WRC Report Number 1773/1-4/12) and "Health risk assessment in connection with the use of microbiologically contaminated source water for irrigation" (WRC Report Number 1226/1/04) have shown that the national water supplies are contaminated with pathogens and may pose a potential risk for the fresh fruit export industry and national safe food supplies. It was argued that a project that focuses on the risks in the supply chain will support and strengthen the findings in WRC Report Number 1773/1-4/12.

The current research team consisted of four groups from the Department of Microbiology and Plant Pathology, University of Pretoria (UP), Department of Food Science, UP, Department of Medical Virology, UP and Department of Microbiology, University of KwaZulu-Natal respectively. The research groups have extensive experience in water microbiology, water quality assessment, waterborne pathogens (including bacteria, viruses and protozoa), method optimization and development for enhanced pathogen detection, food safety, foodborne pathogens, risk assessment of fresh agricultural produce and minimally processed food. In order to do the research with the additional focus beyond the farm gate in the supply chain tasks were allocated according to expertise to achieve the aims as stipulated below.

Aims:

The general and specific aims of this project were:

1. To investigate the links between water quality and microbiological safety in fresh minimally processed and frozen fruit and vegetables.

2. To review the literature study of WRC Report Number 1773/1-4/12 and expand the review to include a comprehensive literature study on processing water quality and its impact on the microbiological safety of food and potential effect on human health.
3. To establish co-operation with the team of researchers involved in WRC project K 5 / 1773/4.
4. To select appropriate sites, with reference to i) Different sources of untreated and treated water (excluding greywater) ii) Different types of microbial contamination iii) Different types of farming – Subsistence, emerging, commercial farming for local and export markets iv) Different crops and produce (specifically fruit and vegetables) v) Different pathways of contamination: water for irrigation and water used for sprays vi) Different pathways of contamination: water as transport and washing medium as part of packing and processing.
5. To propose and standardise the research methodology for the assessment of the impact of contaminated irrigation water and processing water on microbial food safety.
6. To determine the extent (types and quantities) of contamination found in irrigation and processing water at the selected sites, with reference to i) Indicators of water quality ii) Volume of water used for irrigation and processing iii) Microbial contaminants (viruses, bacteria, parasites) iv) Physical parameters (pH, COD, EC, turbidity, temperature, etc.) v) Influence of personal on-site hygiene facilities vi) Influence of external quality control on food safety management at the farming and processing stages of production.
7. To investigate the impact of environmental parameters on the growth kinetics of contaminants, with reference to i) fate and survival ii) Decay rates iii) Surrogate organisms iv) Different irrigation and processing technologies.
8. To study the extent (types and quantities) of contamination found on the irrigated raw produce (fruit and vegetables) up to harvest at selected sites, with reference to i) Microbial contaminants (viruses, bacteria, parasites) – Microbial indicators/index of contamination/risk ii) Method of irrigation, agri-chemical spray water application, etc. iii) Investigate the potential link and/or association between contamination on raw produce and water applied (molecular epidemiology using a reference organism).
9. To study the extent (types and quantities) of contamination found on the raw produce (fruit and vegetables) after harvest, with reference to i) Microbial contaminants (viruses, bacteria, parasites) ii) Microbial indicators/index of contamination/risk iii) Additional contamination through handling, washing, storage, packaging, pre-freezing and minimal processing, etc. iv) Impact of biofilms in processing on product contamination.
10. To make interim and final recommendations for further research, with reference to: Further validation of results and Effective treatment options.

1.2 Description of supply chains for selected fruit and vegetables

A variety of fruit (tomatoes, peaches, pears, strawberries), vegetables (lettuce, spinach, cabbage, onions and broccoli), herbs (parsley and basil) and processed fresh produce (fruit salad and frozen vegetables) were sampled. For a description of the vegetable and fruit supply chains (up to, at and after harvest) included in this study refer to Chapter 1, section 1.2 of Volume 1.

1.3 Selection of appropriate sites and crops for different stages of production and processing

Water and produce samples were collected from selected sites in five provinces in South Africa, i.e. Gauteng (4 sites), Kwazulu-Natal (3 sites), Limpopo (2 sites), North West (3 sites), and the Western Cape (5 sites). Irrigation water sources included water from rivers, boreholes and dams as well as agricultural spray water. Irrigation methods included overhead irrigation (pivot and sprinklers), drip irrigation and micro irrigation. Different types of farming including commercial, small and large scale farms for the local and international export market. Sites where water and produce samples were taken are described in more detail in Chapter 1, section 1.3 of volume 1.

1.4 Standardised research methods for assessment of the impact of irrigation water used during processing and microbial food safety

The aim was achieved by proposing standardised research methods for the assessment of the impact of contaminated irrigation water and processing water on microbial food safety. Methods used by group one; Stellenbosch University (Project 1773) was discussed during the first planning workshop done by group two (Project 1875). It was decided to select all standard ISO methods and methods already published as used by group one (Prof Britz, Prof Buys and Prof Taylor) and to select existing methods already used by the new participants in group two from Prof Korsten and Prof Pillay. In order to transfer the methods and ensure consistency in test methods applied, two week-long training workshops were held: one at KwaZulu-Natal University, Department of Microbiology and one at the University of Pretoria, Departments of Food Science, Virology and Microbiology and Plant Pathology. The standardised research methods were described in more detail in Chapter 1, section 1.4 of volume 1.

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CHAPTER 2

MICROBIAL CONTAMINATION FOUND ON THE RAW PRODUCE (FRUIT AND VEGETABLES) AT HARVEST AS WELL AS AFTER HARVEST

2.1 INTRODUCTION

The demand for fresh convenience foods and the associated increase in foodborne disease outbreaks, linked to increased fresh produce consumption, emphasises the importance of food safety in the supply chain (Jacxsens *et al*., 2009). This motivated governing bodies, the fresh produce industries and food processing companies worldwide to implement control measures to ensure the microbiological safety of all food products. The need for commodity-specific supply-chain management systems and guidelines, based on scientific data (including actual natural microbial levels and pathogen presence/absence) has since been identified (Luning *et al*., 2011).

Subsequently, the microbiological quality as well as contamination and survival potential of human pathogenic bacteria (*Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* Typhimurium and *Staphylococcus aureus* and viruses on fresh produce have been central research questions (Erickson, 2010; Wei and Kniel, 2010; Le Guyader *et al*., 2010; León-Félix *et al*., 2010). Food, which conforms to acceptable bacterial standards, may still be contaminated with viruses (Verhoef *et al*., 2009). In addition to the known risks associated with the prevalence of human pathogens on fresh produce, indicator organisms such as enterococci are emerging as opportunistic pathogens that could cause bacteraemia, nosocomal diseases and endocarditis in humans (Amyes, 2007). Of particular interest is their potential to transfer antibiotic resistance genes to environmental microbes (Johnston *et al*., 2006).

Fresh produce contamination with human pathogens can happen throughout the pre- and post-harvest stages of production and marketing. Potential sources of contamination at the pre-harvest level are soil, faeces, irrigation water, reconstituted fungicides and insecticides, dust, insects, manure, wild or domestic animals (Althaus *et al*., 2012). At the post-harvest level the produce could be contaminated by contact with polluted process water, asymptomatic human carriers, harvesting equipment, and transport containers (Althaus *et al*., 2012).

Due to water scarcity in South Africa, many farmers (rural and commercial) resort to using surface water for irrigation of fresh produce (Nevondo and Cloete, 1999). The microbiological quality of the water is compromised by the presence of informal settlements with inadequate sanitation and access to potable water (Muller *et al*., 2001; Odjadjare and Okoh, 2010). Furthermore, municipal sewage works are unable to cope with the growing demands for efficient treatment plants due to a lack of capacity, accelerated urbanisation rate and increased population, which contribute to the high level of pollution in South African river systems (Chigor and Okoh, 2013; CSIR 2010; Nevondo and Cloete, 1999). Due to the increasing population of immuno-compromised groups (e.g. HIV patients), the presence of human pathogens on postharvest produce poses an even bigger food safety risk compared to

healthy individuals. The risk of illness, especially if contaminated with *E. coli* O157:H7, which has a low infective dose of <100 viable cells, may increase (Doyle and Erickson, 2008). Most *E. coli* O157:H7 outbreaks can be linked directly or indirectly to faecal contamination of water and/or soil from cattle in integrated farming systems and/or humans through poor sanitation practices (Beuchat, 2002).

To date, numerous disease outbreaks caused by the presence of human pathogens (bacteria, viruses and protozoa) on fresh produce have been reported worldwide. Although outbreaks of foodborne disease in humans do occur in South Africa, these incidences are rarely reported (Smith *et al*., 2007). Examples of worldwide foodborne disease included amongst others 1) lettuce associated outbreaks of *S.* Typhimurium (Horby *et al*., 2003), *Salmonella* Newport (Gillespie, 2004), *E. coli* O157:H7 and *Campylobacter jejuni* (http://www.cdc.gov/ecoli/2011/ ecoliO157/romaine lettuce/120711/index.html; accessed 2015 July 21); Heaton and Jones, 2008); 2) tomato associated outbreaks of salmonellosis in 2006 (Behravesh, 2012), *Salmonella* serotype SaintPaul multi-state outbreak in the United States in May 2008 (Mody *et al*., 2011); 3) sprout-associated outbreak of *E. coli* O104:H4 in the European Union in May 2011 (http://www.bbc.co.uk/news/world-europe-13683270; accessed 2015 July 21); 4) spinach-associated outbreaks of *E. coli* O157:H7 (http://www.cdc.gov/ecoli/2006/december/121406.htm; accessed 2015 July 21). *S. aureus*, responsible for causing staphylococcal food poisoning, has also been found to be associated with fresh produce (Harris *et al*., 2003). Onion-associated (including green onion) outbreaks of *Salmonella* spp. occurred in the US (Erickson, 2010). An *E. coli* O157:H7 outbreak during 2008 in Canada was linked to the consumption of fresh red onions (http://foodsafetyinfosheets.files.wordpress.com/2010/03/7-1-09.pdf; accessed 2015 July 21). During the period 1990-2006 noroviruses (NoVs) accounted for 90% of the viral foodborne infections in the US while hepatitis A virus (HAV) accounted for 4% and other enteric viruses for the remaining 6% (Smith De Waal *et al.*, 2008). Berry fruits have been associated with outbreaks of gastroenteritis and hepatitis worldwide (Fiore, 2004; Carter, 2005; Butot *et al*., 2008).

In a previous WRC-funded study the potential link between microbiological quality of irrigation water and at harvest fresh produce was investigated (WRC Report) 1773/1-4/12. This study reported that total coliform and faecal counts for irrigation water and produce at harvest were generally much higher than standards by the World Health Organisation (WHO, 2008) and the Department of Water Affairs (1996). It was concluded that microorganisms on the produce surfaces were present as a result of transfer from irrigation water. Subsequently, WRC project K5/1875/4 was initiated to investigate the potential link between irrigation water and fresh produce (fruit and vegetables) at as well as after-harvest from the farming to the processing stages of production and marketing.

2.2 RESEARCH FINDINGS

2.2.1 GAUTENG PROVINCE – Microbiological quality (including virological analysis) of parsley from a farm in the Elandsdrift area

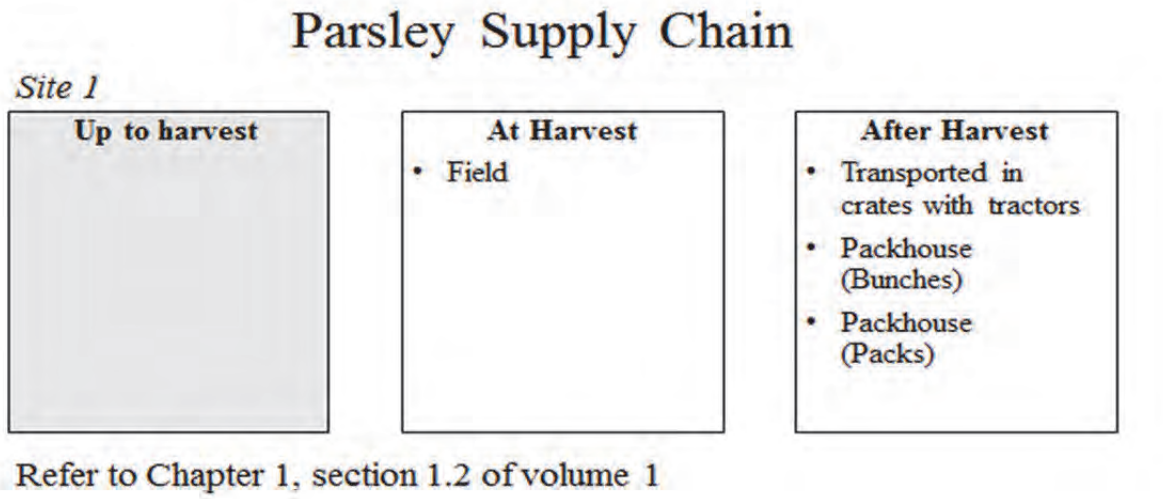
Specific aim

This study aimed to address the lack of scientific knowledge regarding the microbiological quality of fresh herbs at harvest and after harvest and the potential impact of irrigation water quality on fresh produce safety.

Experimental procedures

Description of sampling site

This study was conducted during 2011 on a vegetable/herb production farm in Gauteng using the Crocodile River for irrigation purposes.



Sampling and processing

Parsley samples (replicates of 3) were collected during two field trips in 2011 from two fields, from bunches in the packhouse and after packaging. Ten grams of each sample (parsley leaves) were weighed into a sterile polyethylene bag containing 90 mℓ of 0.1% Peptone Water Buffered solution and homogenised.

Microbiological analysis

Decimal dilutions of filter membranes were prepared as described by Standing *et al*. (2013) and plated onto Standard 1 agar (STD 1) and malt extract agar (MEA) to determine total viable bacteria and fungi/yeast numbers. Plates were incubated at 25°C for two (STD 1) and five days (MEA) respectively. The total coliform numbers and presence of *Escherichia coli*, were determined by using a commercially available Colilert test kit according to the manufacturer's instructions (IDEXX Laboratory Inc., One IDEXX Drive, Westbrook, USA, Part no. 98-08877-00). Each of the parsley samples were enriched for detection of human pathogenic bacteria by incubation at 37 °C for 24 hours, before multiplex PCR analysis.

Samples enriched for determining the presence of *E. coli* (including *E. coli* O157:H7) by incubating the homogenised parsley samples at 37°C for 24 hours followed by streaking onto Eosin methylene blue differential medium (EMB) (Merck) for detection of typical *E. coli* colonies. *Salmonella* spp. were detected by enriching each of the water samples in Salmonella enrichment broth for 24 hours at 37°C, followed by streaking onto Xylose lysine deoxycholate (XLD) agar for detection of typical colonies. *Listeria* spp. was enriched in Listeria enrichment broth at 37ºC for 24 hours, where after it was plated onto Listeria agar (all enrichment media were obtained from Oxoid, Johannesburg). All presumptive *E. coli*, *Salmonella* and *Listeria* isolates from selective media were purified by re-streaking onto selective media and identified using PCR analysis and Matrix Assisted Laser Ionisation-Time of Flight (MALDI-TOF).

Determination of *E. coli* O157:H7, *S.* Typhimurium, *Listeria monocytogenes* and *Staphylococcus aureus* presence

DNA was extracted from each of the enriched produce samples using the Quick-gDNA miniprep kit (Zymo Research, California, USA). Multiplex PCR analysis was performed using the DNA as template using pathogen specific primers (Cebula *et al*., 1995; Standing *et al*., 2013).

Viral recovery

Irrigation water: Prior to viral recovery the water sample was seeded with a known titre of mengovirus which was used as a process control. Viruses were recovered from the 10 ℓ irrigation water samples using a glass wool adsorption-elution procedure based on the method of Vilaginès *et al*. (1993) as described by Wolfaardt *et al*. (1995) and Vivier *et al*. (2004). Viruses were further concentrated to a final volume of 10 mℓ using a modification of the polyethylene glycol /sodium chloride (PEG/NaCl) precipitation method originally described by Vilaginès *et al*. (1997) and Minor (1985).

Virus Detection Methods

Nucleic acid extraction: Viral nucleic acid was extracted using two different technologies. Genomic viral nucleic acid was extracted from 1 mℓ of the recovered virus concentrate using the Nuclisens EasyMAG automated magnetic extraction platform (bioMérieux SA, Marcy l'Etoile, France) following the manufacturer's instructions and from a second 1 mℓ of the recovered virus concentrate using the MagNA Pure LC Total Nucleic Acid Isolation Kit (large volume) (Roche Diagnostics GmbH, Mannheim, Germany) in a MagNA Pure LC Robotic instrument (Roche), following the manufacturer's instructions. Purified nucleic acid was aliquoted and stored at -70°C.

Viral amplification and detection

The NoVs, HAV and mengovirus were detected using optimised qualitative rt RT-PCR assays based on TaqMan technology and published primers and probes, namely: HAV (Costafreda *et al*., 2006); NoV GI (da Silva *et al*., 2007; Svraka *et al*., 2007); NoV GII (Kageyama *et al*., 2005; Loisy *et al*., 2005) and mengovirus (Pintó *et al*., 2009). Briefly complementary DNA (cDNA) was prepared using RevertAid™ Premium Reverse Transcriptase (Fermentas Life

Sciences, Burlington, Ontario) and amplification was performed using the EXPRESS qPCR Universal Mix (Invitrogen, Carlsbad, CA) in a final volume of 20 µℓ. The reaction was carried out in sealed glass capillaries in a Lightcycler® v2.0 instrument (Roche). An amplification control (AC), which was designed and constructed as part of Project K5/1773//4, was included in each NoV GII reaction. An extraction negative control (nuclease-free water; Promega Corp., Madison, WI), a rt RT-PCR negative control and a *rt* RT-PCR positive control, which comprised of RNA from the target virus, were included in each assay.

Results

Microbiological analysis

Parsley:

ACC counts – The total aerobic counts on the parsley varied between 2.8 log CFU/g (minimum) and 6.7 log CFU/g (maximum) (Table 1).

Fungi and Yeasts – minimum of 2.4 log CFU/g and maximum 5.2 log CFU/g, ranging mostly between 4 log CFU/g and 5 log CFU/g.

E. coli 0157:H7 – not detected (Table 2).

L. monocytogenes – not detected.

S. Typhimurium – not detected.

Norovirus GI – not detected.

Norovirus GII – detected on parsley samples from the field and packhouse.

Table 1 Summary of results of microbiological and viral analysis of parsley

Produce	**Field trip one**		**Field trip two**	
Parsley	**ACC log CFU/g**	**Fungi log CFU/g**	**Bacteria log CFU/g**	**Fungi log CFU/g**
Field sample 1	5.81	4.16	5.12	4.63
Field sample 2	6.32	4.60	5.67	4.89
Pack house (bunches)	6.75	5.18	2.75	2.38
Pack house (packs)	5.78	4.24	5.20	4.37

Table 2 Human pathogenic bacteria and viral presence/absence in parsley samples

Produce	**Human pathogenic bacteria**				**Viruses**			
Parsley	***E. coli* O157:H7**	***S.* Typhimurium**	***L. monocytogenes***	***S. aureus***	**NoVG 1**	**NoVG II**	**HAV**	**Mengo virus**
Field 1	ND	ND	ND	ND	ND	POS	ND	POS
Field 2	ND	ND	ND	ND	ND	POS	ND	POS
Packhouse (bunches)	ND	ND	ND	ND	ND	POS	ND	POS
Packhouse (packs)	ND	ND	ND	ND	ND	POS	ND	POS

ND=None detected; Pos = Positive for pathogen presence

Discussion and conclusion

Current microbiological specification guidelines for ready-to-eat fresh fruit and vegetables require coliform counts of less than 200/g, yeast and mould counts of < 100 000 (log 4)/g, *E. coli* 0/g and *Salmonella* 0/25 g (DoH, 2000). The yeast and mould counts for parsley were mostly higher than the specifications. Although the human pathogenic bacteria tested for were not detected in the parsley samples, the presence of Norovirus GII on the parsley samples from the field and in the packhouse could pose a potential health risk, since parsley is often consumed raw. Sixty percent of foodborne disease outbreaks linked to leafy green vegetable consumption between 1973 and 2006 was caused by Norovirus, compared to only ten percent caused by *Salmonella* spp. and nine percent by *E. coli* (http://www.sciencedaily.com/releases/2008/03/080317164356.htm; accessed 2015 21 July).

2.2.2 GAUTENG PROVINCE – Microbiological quality and safety of lettuce produced from a farm in the Elandsdrift area

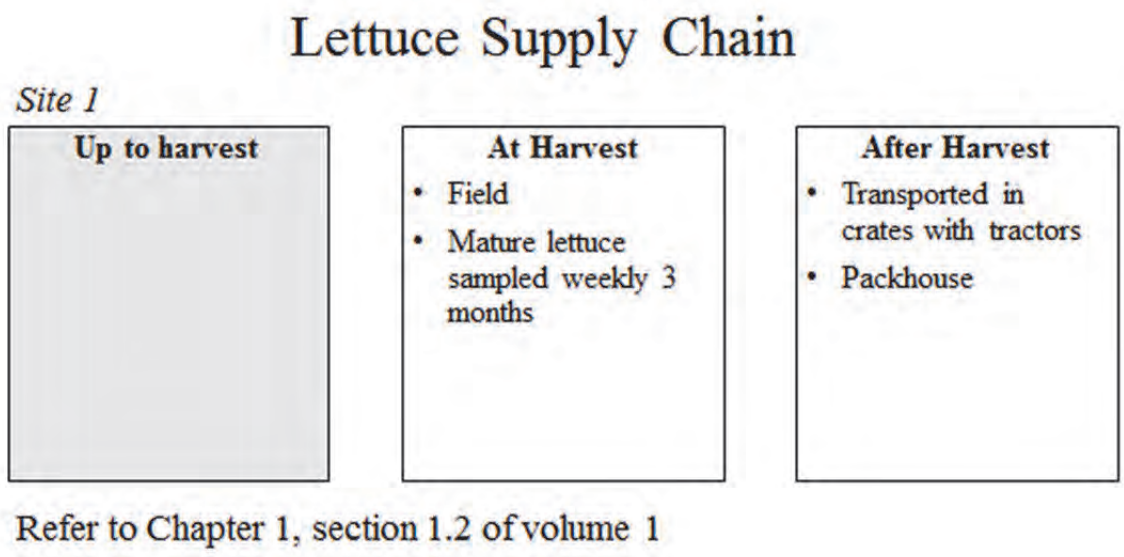
Specific aim

The main objectives were to monitor the microbiological quality of irrigation water (dam, pump house and from irrigation pipes) and the potential impact on the microbiological quality and safety of lettuce.

Experimental procedures

Sampling site

A commercial farm on the banks of the Crocodile River in the Crocodile (West) Marico Water Management Area, in Gauteng was selected. River water on the farm is collected in a temporary holding dam which is then pumped and distributed to different areas of the farm through the pump house. The water the crops received was therefore freshly pumped from the main holding dam into which the river flowed.



Sampling and processing

Lettuce: Winter Supreme lettuce seedlings (*Lactuca sativa L.* var. *capitata*) were sprouted on the farm and transplanted into the field. Approximately 15 seedlings were collected with their root systems and rhizospheres 1 week before being transplanted into the field; this sampling day was considered as week 0. Lettuce phyllosphere, root and rhizosphere samples were then collected every week for 12 weeks including at harvest, in the pack house (after harvest) and at a retail store. Fifteen randomly selected lettuce plants were collected for each sampling period and transported to the laboratory in a cooler-box for further analysis within 12 hours after collection. Lettuce heads were separated aseptically from their root systems and placed in a sterile bag for further analysis. The rhizosphere was then removed from the root systems into a sterile bag by shaking and the roots were then transported in their own sterile bag for further analysis. The outermost leaves of the lettuce heads were aseptically removed and discarded whilst samples were collected from the portion of the head that was considered to be the middle. Samples from 5 separate heads were collected and combined to make up a single replicate of 10 g; three replicates in total were used. Samples from 5 separately collected root systems and rhizospheres were also combined respectively, to make up a single replicate of 10 g; three replicates in total were used for both the root systems and the rhizosphere samples. No root or rhizosphere samples were collected from the pack house or retail samples. The same batch of lettuce was followed from the seedling stage all the way to the retail store with the logistical assistance of the farm manager.

Once the phyllosphere, root and rhizosphere samples were combined into replicates, each replicate was placed in a sterile stomacher bag with 90 mℓ 0.1% peptone buffered water (Merck, South Africa). Phyllosphere and root samples were then stomached for 2 min at 230 rev min^{-1} in a Seward Stomacher 400 Circulator (Seward Ltd., London, UK). Rhizosphere samples were sonicated in an ultrasonic bath for 2 min at room temperature. Serial dilutions

were then performed for all of the samples respectively. Cells were plated and incubated as previously discussed. Colonies were recorded and transformed to log (x + 1) CFU g^{-1}.

Nucleic acid extraction and Polymerase Chain Reaction for pathogen detection-
Filters from the water samples and the leaves, roots and soil samples were enriched in Tryptone Soy Broth for 24h at 37°C. DNA was extracted from each sample using a Zymo Quick-gDNA MiniPrep kit (Inqaba Biotech, Johannesburg, South Africa) as per the manufacturer's instructions. *Escherichia coli* and *Escherichia coli* O157:H7 were detected using the primers: Eco1 5'-GACCTCGGTTTAGTTCACAGA-3', Eco 2 5'-CACACGCTGACGCTGACCA-3' (Schippa *et al.,* 2010) and UidAa 5'-GCG AAA ACT GTG GAA TTG GG-3', UidA 5'-TGA TGC TCC ATA ACT TCC TG-3'. A universal 16S reaction was also included in the multiplex PCR reaction as an internal PCR control using the following primers: 27 forward 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492 reverse 5'-ACGGCTACCTTGTTACGAC-3'primers. The PCR amplification was carried out in a GeneAmp 2400 PCR system (Applied Biosystems, Foster City, USA) using Takara EmeraldAmp Max HS PCR Master Mix (Separations, Johannesburg, South Africa). The PCR mixture contained: 25µl EmeraldAmp MAX HS PCR Master Mix; 0.2µM each of Eco 1, Eco 2, UidAa, UidAb, 27 forward and 1492 reverse respectively; 80ng DNA Template; and sterile distilled water. PCR was performed for 30 cycles under the following conditions: 10s at 98˚C, 30 sec at 60˚C and 1 min/kb at 72˚C. The products of the amplification were then analysed by electrophoresis in a 2% (w/v) agarose gel containing 0.01% Ethidium bromide (Merck).

Statistical analysis
Data (log (x + 1) CFU g^{-1} for lettuce leaf, root and soil samples was analysed using an analysis of variance (ANOVA) with SAS-9.2 software (SAS Institute Inc., Cary, United States of America). Means obtained were compared by using the Fishers protected least significant difference (LSD) test at a 5% (P = 0.05) level of significance.

Results
Results obtained for the lettuce phyllosphere, roots and soils are summarised in Table 3.

Table 3 Number of heterotrophic bacterial plate counts (Log_{10} (x + 1) CFU g^{-1}) on lettuce phyllosphere, root surfaces and rhizospheres

Time (weeks)	Phyllosphere	Roots	Rhizosphere
0	5.386^{LMN}	5.459^{KLM}	4.5273^{OP}
1	6.6259^{BCDEF}	6.8922^{BC}	7.3519^{A}
2	6.4486^{CDEF}	7.0447^{AB}	6.9128^{ABC}
3	5.8151^{HIJKL}	7.0598^{AB}	5.8806^{GHIJK}
4	6.1944^{FGHI}	4.9667^{NO}	6.6933^{BCDE}
5	5.5141^{JKLM}	6.8366^{BC}	6.3408^{DEFG}
6	5.9624^{GHIJ}	6.5047^{CDEF}	6.7202^{BCDE}
7	5.6506^{JKL}	6.7911^{BCD}	6.2568^{EFGHI}
8	5.8458^{HIJKL}	6.6665^{BCDEF}	6.2748^{EFGH}
9	5.1179^{MN}	6.6116^{BCDEF}	6.5120^{CDEF}
10	5.4161^{KLMN}	6.6964^{BCDE}	6.3517^{DEFG}
11	5.0837^{MN}	6.8496^{BC}	6.6204^{BCDEF}
12	4.3638^{P}	5.6765^{JKL}	6.7936^{BCD}
At harvest	5.6705^{JKL}	6.6264^{BCDEF}	5.6046^{JKL}
After harvest	4.2869^{P}	N/A	N/A
Retail samples	5.7898^{IJKL}	N/A	N/A

All means obtained from three replicates. All means followed by the same letter are not significantly different ($P < 0.05$).

None of the phyllosphere samples (ranging from seedlings to retail samples) tested over the 12 week period showed the presence of any form of *E. coli* (Table 4). Root samples from week 2, 6, 7and 9 showed positive results for general *E. coli* but the pathogen was only detected in the soil on week 3 and 6. *E. coli* O157:H7 was not detected in any of the samples that tested positive for the presence of general *E. coli.*

Table 4. Presence/ absence of *E. coli* and *E. coli* O157:H7 in lettuce and water samples

Sample	Time (weeks)													
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**After Harvest**
Phyllosphere	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)
Roots	- (-)	- (-)	+ (-)	- (-)	- (-)	- (-)	+ (-)	+ (-)	- (-)	+ (-)	- (-)	- (-)	- (-)	- (-)
Rhizosphere	- (-)	- (-)	- (-)	+ (-)	- (-)	- (-)	+ (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)
Dam	- (-)	+ (-)	- (-)	+ (-)	+ (-)	+ (-)	+ (-)	+ (-)	+ (-)	+ (-)	- (-)	- (-)	+ (-)	+ (-)
Pump house	+ (-)	+ (-)	- (-)	+ (-)	+ (-)	+ (-)	+ (-)	+ (-)	+ (-)	+ (-)	- (-)	- (-)	+ (-)	+ (-)
Irrigation pipe	- (-)	- (-)	- (-)	+ (-)	+ (-)	+ (-)	+ (-)	+ (-)	+ (-)	+ (-)	+ (-)	- (-)	+ (-)	- (-)

E. coli results shown with *E. coli* O157:H7 results shown in parenthesis

“+” Positive result via PCR

“-” Negative result via PCR

Discussion and conclusion

Due to the potential for crop contamination by pathogenic microorganisms through the application of contaminated water (Castro-Ibáñez *et al*., 2015; Chitarra *et al*., 2014; Holvoet *et al*., 2013) the likelihood of lettuce contamination by general *E. coli* and *E. coli* 0157:H7 was studied during primary production on a working farm in the current study. In a study by Solomon *et al.* (2002) it was found that more than 90% of spray-irrigated lettuce showed signs of *E. coli* 0157:H7 contamination, whereas less than 20% of surface-irrigated lettuce did. As spray irrigation was used in the current study, the lettuce crops were at a higher risk for contamination, yet none of the lettuce samples collected showed signs of *E. coli* contamination.

Islam *et al*. (2004) found that soil often has high levels of indicator organisms and that these can also contribute to additional lettuce contamination especially during seasons when high rainfall is experienced, as splash back often occurs. Studies by Solomon *et al*. (2002) and Franz *et al*. (2007) showed that *E. coli* O157:H7 from contaminated irrigation water was able to migrate directly from the soil surrounding the root system to the edible portion of lettuce plants through the root conducting tissues. In the current study, 4 of the root samples tested positive for *E. coli*, whereas only two of the soil samples tested positive. Despite these samples showing positive results, the *E. coli* did not persist in those environments as have been demonstrated previously (Islam *et al*., 2004). Islam *et al.* (2004) showed that *E. coli* O157:H7 was able to persist in high numbers for >5 months after the original application of contaminated irrigation water. They also showed that the soil used in their study played a role in the survival of *E. coli* O157:H7, as contamination of crops without constant direct exposure to *E. coli* O157:H7 still occurred.

When taking into account the entire "farm to plate" chain, microbial contamination of the crops can occur at any point from vegetable production and harvesting to packing (Islam *et al*., 2004). A study in Pakistan by Ensink *et al*. (2007) showed that contamination did not only occur through the application of poor quality irrigation water, as the authors found that vegetables collected from the local market often showed higher *E. coli* concentrations when compared to the same furrow-irrigated vegetables collected from the fields. Irrigation water quality therefore plays an important role in the quality of crops, but other factors such as post-harvest handling should also be considered.

2.2.3 GAUTENG PROVINCE – Virological analysis of strawberry samples from a farm in the Pyramid district, Tshwane Metropolitan area

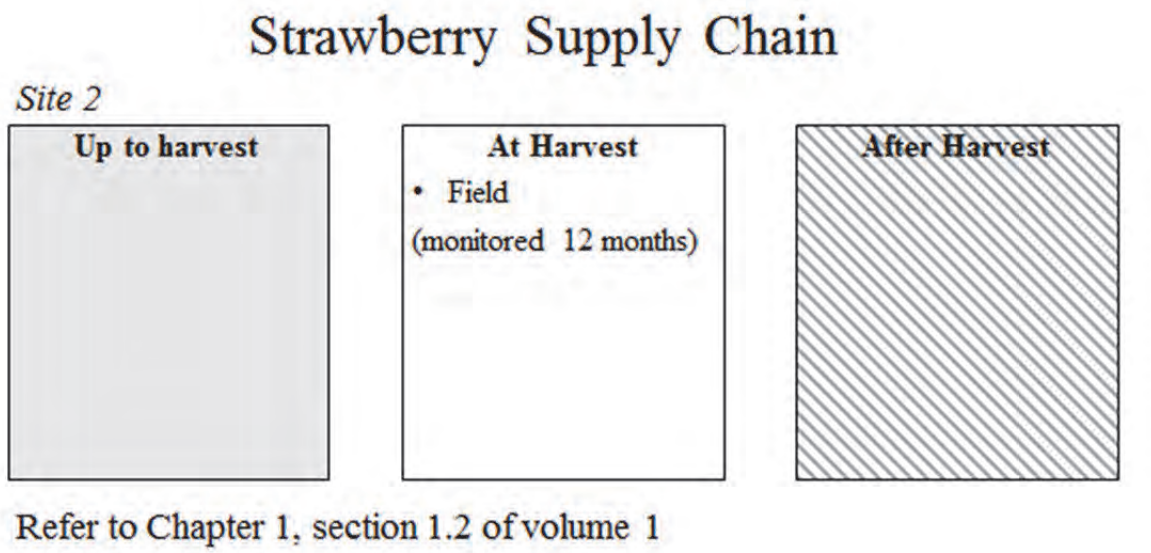
Specific aim

The aims of this study were; i) to assess the quality of surface water used for irrigation of strawberries, ii) to assess the sensitivity of two extraction procedures, iii) to determine the presence of selected enteric viruses (NoVs and HAV) on strawberries and in irrigation water(s) used and, iv) to characterise and determine the phylogenetic relationship of the viruses detected.

Experimental procedures

Sampling site

A small-scale strawberry farmer to the north of Pretoria, Gauteng, was approached for the collection of strawberries, when in season or available, and irrigation water from the irrigation canal flowing from the Bon Accord dam used for the irrigation of the strawberries. It was noted that the farmer used overhead irrigation at the start of the study but changed to drip irrigation soon thereafter when he became aware of preliminary test results.



Strawberry sampling

From September 2010 to August 2011 irrigation water samples were collected on a monthly basis from an irrigation canal flowing from the Bon Accord Dam, Pretoria. Water samples were collected in sterile containers (10 ℓ with an additional 1 ℓ) and transported in a cooler bag to the laboratory and stored at 4°C until processed.

To obtain a representative sample size, three samples, each comprising of three strawberries, were sampled from different areas of the field and analysed individually. Each strawberry was collected in a separate zip lock bag and gloves were changed between the picking of each strawberry to prevent possible cross contamination. The samples were placed in a cooler bag and transported to the laboratory and stored at 4°C until processed. The leaves from the base of the strawberries were removed before proceeding with viral recovery.

Analysis for indicator organism

Thermotolerant (faecal) coliforms

Thermotolerant (faecal) coliforms were enumerated from a 100 mℓ aliquot of sampled irrigation water (1 ℓ) using a membrane filtration method (0.45 µm Sartorius Stedim filters). Turbid water samples that could possibly restrain or inhibit the flow of the water through the filter were diluted ten-fold using Modified Scholtens' Broth (MSB). Faecal coliform selective agar plates (m-FC agar plate (Selecta Media, Quantum Biotechnologies [Pty] Ltd, Ferndale,

SA) were used, onto which the filters were placed and incubated overnight at 44.5°C. Individual blue colonies on the surface of the filter(s) were used to calculate the number of colony forming units (CFU)/100 mℓ present for each water sampled. In cases where individual colonies could not be counted due to the higher number present/over growth, serial ten-fold dilutions of the water were made with PBS (Sigma-Aldrich Co.) and the enumeration process repeated.

Escherichia coli

The process for the enumeration of *E. coli* from the irrigation water samples was identical to that for the thermotolerant (faecal) coliforms with the following differences; i) a 0.45 µm filter from Merck Millipore (Merck) was used, ii) m-ColiBlue24 *E. coli* specific broth (Merck) was used and iii), the plates were incubated overnight at 35°C. The CFU/100 mℓ was calculated by counting the individual blue colonies present. Serial ten-fold dilutions of water were made in cases where individual colonies could not be enumerated.

Viral Recovery from irrigation water and strawberries and virus detection were done as described for parsley in section 2.2.1.

Characterisation of Noroviruses

For the characterisation of NoVs, a highly conserved region at the 5'end of the NoV capsid gene was amplified and sequenced. A two-step conventional PCR amplification was performed with published sets of primers. The KAPA Taq Hot Start PCR kit (KAPA Biosystems, Cape Town, SA) with 5 mM dNTP's (Fermentas Life Sciences), 37.5 mM magnesium chloride (KAPA Biosystems), and 5 µℓ of cDNA was used for the first round of amplification. For NoV GI, primer set 1 (Table 5) and for NoV GII primer set 3 (Table 5) with 25 µM and 50 µM forward and reverse primer concentration, respectively, was used. The reaction mix was prepared to a final volume of 20 µℓ. Amplification was performed on a Little Genius thermal cycler (BIOER Technology, Hangzhou, China) using the following cycling conditions; pre-denaturation at 94°C for 10 min, followed by 45 cycles of 94°C for 30 sec, 50°C (NoV GI) or 55°C (NoV GII) for 45 sec, 72°C for 1 min, followed by a final extension step at 72°C for 5 min. A semi-nested PCR protocol was used for the second round of amplification. AmpliTaq® Gold DNA polymerase (0.025 U/µℓ) (Applied Biosystems) with 5 mM dNTPs (Fermentas Life Sciences), and 1 µℓ of the first round PCR product was used. For NoV GI, primer set 2 (Table 5) and for NoV GII primer set 4 (Table 5) with 5 µM and 25 µM forward and reverse primer concentrations, respectively, was used. Amplification was identical to that of the first round. The PCR products were analysed by 2% agarose gel electrophoresis (SeaKem® LE Agarose, Lonza, Rockland, NY) and visualised by EtBr staining and UV illumination using a 100 bp marker (O'GeneRuler, Fermentas Life Sciences, Burlington, Ontario, Canada) to determine the band size(s).

Table 5 Primer sequences used for genotyping of NoV GI and NoV GII.

Virus	Primer Set	Primer		Reference
		Forward	Reverse	
Norovirus GI	1	QNIF4	G1SKR	Da Silva *et al*. (2007) and Kojima *et al*. (2002)
	2	G1SKF	G1SKR	Kojima *et al*. (2002)
Norovirus GII	3	QNIF2	G2SKR	Loisy *et al*. (2005) and Kojima *et al*. (2002)
	4	G2SKF	G2SKR	Kojima *et al*. (2002)

Cloning of PCR products

Amplified PCR fragments of approximately 300-400 bp were recovered from the gel and concentrated to 10 µℓ using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corp., Irvine, CA). The purified PCR fragments were cloned into a plasmid vector using the CloneJET™ PCR cloning kit (Fermentas Life Sciences) according to the manufacturer's recommendations. Four microliter of the ligation reaction was used to transform the *E. coli* competent cells (10 µℓ) (Lucigen Corp., Middleton, WI). The transformed *E. coli* cells were incubated at 37°C for 1 hour in 900 µℓ recovery media (Lucigen Corp.) under vigorous shaking. A 100 µℓ aliquot of the suspension was plated on LB enriched agar containing 100 µg/mℓ ampicillin (Sigma-Aldrich Co.) and incubated overnight at 37°C. Ten individual colonies were randomly isolated from the plates and screened for the specific insert by PCR amplification. GoTaq® Flexi DNA Polymerase (5u/µℓ) (Promega Corp.) with 2.5 mM dNTP's (Fermentas Life Sciences) and 4 µM pJET 1.2/blunt specific primers according to the manufacturer's recommendations was used. Amplification was performed on a Little Genius thermal cycler (BIOER Technology) using the following cycling conditions; pre-denaturation at 95°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min with a final extension step at 72°C for 10 min. The PCR fragments were analysed by electrophoresis in a 2% agarose gel (SeaKem® LE Agarose) and visualised under UV illumination after staining with EtBr. A 100 bp marker (O'GeneRuler) was used to determine the band size(s).

An additional ten clones were sequenced in those samples where NoV could be typed. The amplification process were repeated for those samples in which an appropriate fragment size could not be obtained with changes made to the nucleic acid concentration (serial tenfold dilution series), the Taq polymerase used for the first and second round amplification steps (KAPA Taq vs. AmpliTaq Gold), the volume of PCR product used for second round amplification (1 vs. 2 µℓ) and the cycling conditions of the PCR assays were adjusted.

Sequencing and sequence analysis of PCR products

Amplified PCR products were purified using the DNA Clean & Concentrator™-25 Kit (Zymo Research Corp.) and eluted in 10 µℓ. The fragments were sequenced based on the Sanger sequencing method using the ABI Prism BigDye® Terminator v3.1 Cycle sequencing Kit

(Applied Biosystems) and pJET 1.2/blunt specific primers (3.2 pmol) according to the manufacturer's recommendations. A 2 µℓ aliquot of the purified PCR fragments were used in each PCR reaction. Amplification was performed on a Little Genius thermal cycler (BIOER Technology) under the following cycling conditions; pre-denaturation at 94°C for 3 min followed by 25 cycles of 94°C for 30 sec, 50°C for 10 sec and 60°C for 4 min. The ABI 3130 automated analyser (Applied Biosystems) was used to analyse the amplified PCR products. Sequencer™ v4.10.1 and MEGA version 6 (Tamura *et al*., 2011) was used for the analysis of the sequences Norovirus reference were strains obtained from (http://0-blast.ncbi.nlm.nih.gov.innopac.up.ac.za/) (Table 6). MAFFT version 6 (http://0-mafft.cbrc.jp.innopac.up.ac.za/alignment/server/index.ht) was used to align the sequences. After manual adjustments of the alignment, phylogenetic analysis was performed with MEGA version 5.1 using the neighbour-joining method. Genotypes were assigned based on the clustering of the individual samples in the phylogenetic tree with a bootstrap support of >70%.

Table 6 Norovirus reference strains used in phylogenetic analysis of field strains.

Genotype	GenBank Accession no.	Strain	Year	Country of Origin
GII.1	HCU07611	Hawaii	1971	United States
GII.2	X81879.1	Melksham	1994	United Kingdom
GII.3	GU980585.1	CBNU1	2006	Korea
GII.3	HM635200.1	Seoul	2009	Korea
GII.4	X76716.1	Bristol	1993	United Kingdom
GII.5	AJ277607.1	Hillingdon	1990	United Kingdom
GII.6	AJ277620.1	Seacroft	1990	United Kingdom
GII.7	AJ277608.1	Leeds	1990	United Kingdom
GII.8	AF195848.1	98-18	1998	Amsterdam
GII.9	AY038599.2	VA97207	1997	United States
GII.10	AF427118.1	Erfurt	2000	Germany
GII.11	AB074893.1	Sw918	1997	Japan
GII.12	AJ277618.1	Wortley	1990	United Kingdom
GII.13	AY113106.1	Fayetteville	1998	United States
GII.14	AY130761.1	M7	1999	United States
GII.15	AY130762.1	J23	1999	United States
GII.16	AY502010.1	Tiffin	1999	United States
GII.17	AY502009.1	CS-E1	2002	United States
GII.18	AY823304.1	OH-QW101	2003	United States
GII.19	AY823306.1	OH-QW170	2003	United States
GII/Unknown	HM560937.1	205	2010	Taiwan

Viral detection from paired irrigation water and strawberry samples

From September 2010 to August 2011 a total of 24 samples comprising of 72 strawberries in total and 12 irrigation water samples were collected and analysed for the presence of NoV GI,

NoV GII and HAV (Table 7). Possible PCR inhibitors were removed by diluting the nucleic acid ten-fold prior to amplification. Norovirus GI, NoV GII and HAV were detected in 8.3% (1/12), 41.7% (5/12) and 0% (0/12), respectively, of the irrigation water samples. Similar results were noted in the strawberry samples with an overall detection percentage of 4.2% (1/24), 20.8% (5/24) and 4.2% (1/24) recorded for NoV GI, NoV GII and HAV, respectively.

Table 7 Summary of results for viral analysis using DNA extracted with two different nucleic-acid-extraction-isolation commercially available kits

Sample	Sampling date	Real-time RT-PCR analysis					
		NoV GI		NoV GII		HAV	
		easy MAG	MagNA Pure	easy MAG	MagNA Pure	easy MAG	MagNA Pure
Irrigation water	10.09.22	+	-	-	-	-	-
Strawberry (a)		-	-	-	+	-	-
Strawberry (b)		-	-	-	-	-	-
Strawberry (c)		-	-	-	+	-	-
Irrigation water	10.10.25	-	-	+	+	-	-
Strawberry (a)		-	-	-	-	-	+
Strawberry (b)		-	-	-	-	-	-
Strawberry (c)		-	-	-	-	-	-
Irrigation water	10.11.22	-	-	+	-	-	-
Strawberry (a)		-	-	-	-	-	-
Strawberry (b)		-	-	-	-	-	-
Strawberry (c)		-	-	-	-	-	-
Irrigation water	10.12.15	-	-	-	-	-	-
Strawberry (a)		-	-	-	-	-	-
Strawberry (b)		-	-	-	-	-	-
Strawberry (c)		-	-	-	-	-	-
Irrigation water	11.01.21	-	-	+	+	-	-
Irrigation water	11.02.22	-	-	-	-	-	-
Strawberry (a)		-	-	-	-	-	-
Strawberry (b)		-	-	-	-	-	-
Strawberry (c)		-	-	-	-	-	-
Irrigation water	11.03.22	-	-	-	-	-	-
Irrigation water	11.04.21	-	-	-	-	-	-
Irrigation water	11.05.21	-	-	-	-	-	-

The use of optimised multiplex *rt* RT-PCR assays allowed for the detection of the above and additional viral species (Table 8). Norovirus GI, NoV GII and HAV could be detected in 9.1% (1/11), 36.4% (4/11) and 0% (0/11) in irrigation water samples with the singleplex *rt* RT-PCR assays, respectively. However, with the multiplex *rt* RT-PCR assays NoV GI, NoV GII and HAV was detected in 27.3% (3/11), 27.3% (3/11) and 0% (0/11) of the irrigation water samples, respectively. An additional three NoV GI positive irrigation water samples were detected by the multiplex *rt* RT-PCR assay. Human AstV, HRV and SaV were detected

in 36.4% (4/11), 54.5% (6/11) and 9.1% (1/11) of the irrigation waters collected. Mengovirus was consistently detected by both assays.

Phylogenetic analysis

The 5' end of the NoV GII capsid gene (273 bp) was used in the phylogenetic analysis. The NoV GII strains could be genotyped from three of the 10 positive samples. However, no strains from the five NoV GI positive samples could be genotyped (Fig. 1). A neighbour-joining tree using the amino acid sequence alignments of the reference NoV GII strains (Table 6) and unknown NoV strains from this study was drawn (Fig. 1). Phylogenetic analysis could closely match the NoV strain in the irrigation water as swine NoV GII.18 and that of the strawberry as human NoV GII.7. BLAST results of the irrigation water strains found an unidentified NoV GII strain (Table 9) recovered from an oyster in Taiwan as the closest match (99% nucleotide sequence identity) (Fig. 1) (Table 10). Analysis of this sequence strain and that of the individual irrigation water sequences closely grouped together with swine NoV GII.18. A pairwise alignment of the amino acid and nucleotide sequences of the Jul/Aug Bon Accord Irrigation Water (BIW) clones, the swine NoV GII.18 and the NoV strain from the oyster (Table 9) revealed an overall 81.8% amino acid and 76.3% nucleotide similarity of the clones to the swine NoV GII.18 strain (Table 10). An overall 99.5% amino acid and a 93.47% nucleotide sequence identity existed between the Jul/Aug BIW clones and that of the oyster NoV GII strain. In the majority of the Jul/Aug BIW clones, insignificant changes were noticed between the NoV GII strain collected in July and that in August 2011 (Fig. 1) (Table 10). Separate groupings of two clones (Gauteng Province (GP) July BIW clone 4 and GP July BIW clone 4) were noticed but these differences were not supported by bootstrap analysis. Similarly, Bon Accord Strawberries (BST) clones 3 and 7 (Fig. 1) grouped together but separate from the other clones, yet the differences noticed were not supported by bootstrap analysis.

Table 8 Summary of results from the comparison of singleplex and multiplex *rt* RT-PCR assays for detection of selected enteric viruses

Sample	Sample date	Singleplex rt RT-PCR* assay				Multiplex rt RT-PCR* assays							Total viruses detected
		HAV	NoV GI	NoV GII	PC	HAV	NoV GI	NoV GII	PC	HAstV	HRV	SaV	
Irrigation water	2010.09.22	-	+	-	-	-	-	-	-	-	-	-	1
	2010.10.25	-	-	+	-	-	+	+	-	-	-	-	3
	2010.11.22	-	-	+	-	-	-	-	-	+	-	-	2
	2010.12.15	-	-	-	-	-	+	-	-	+	+	-	3
	2011.02.22	-	-	-	-	-	-	-	-	+	-	-	1
	2011.03.22	-	-	-	+	-	-	-	+	-	-	+	2
	2011.04.21	-	-	-	+	-	-	-	+	-	-	-	-
	2011.05.21	-	-	-	+	-	-	-	+	+	+	-	2
	2011.06.21	-	-	-	+	-	-	-	+	-	-	-	-
	2011.07.26	-	-	+	+	-	+	+	+	-	-	-	3
	2011.08.24	-	-	+	+	-	-	+	+	-	-	-	2

Table 9 Summary of genotyping results of NoV GI and NoV GII strains detected in the irrigation water and on strawberries

Sample	Date	Norovirus genogroup	Genotyping
Irrigation water	2010.09.22	GI	Untypable
	2010.10.25	GII	Untypable
	2010.10.25	GI	Untypable
	2010.11.22	GII	Untypable
	2010.12.15	GI	Untypable
	2011.01.21	GII	Untypable
	2011.07.26	GII	GII.18
	2011.07.26	GI	Untypable
	2011.08.24	GII	GII.18
Strawberries	2010.09.22	GII	Untypable
	2010.09.22	GII	Untypable
	2011.06.21	GII	Untypable
	2011.06.21	GII	Untypable
	2011.08.24	GII	GII.7
	2011.08.24	GI	Untypable

Table 10 Pairwise analysis of amino acid and nucleotide sequences of four swine NoVGII.18 strains, with two additional clones of each of the samples respectively, were used to determine the relatedness of the strains to each other and to the two swine NoVGII.18 reference strains (AY823304 and HM560937). A 306 bp region of the NoVGII capsid protein was aligned and used for analysis.

		AY823304	HM560937	Jul 11 BIW				Aug 11 BIW				
		Swine	Oyster	Clone 1	Clone 2	Clone 3	Clone 4	Clone 1	Clone 2	Clone 3	Clone 4	Clone 5
AY823304 Swine			82.18	82.18	82.18	83.17	81.12	82.18	82.18	82.18	79.21	82.18
HM560937 Oyster		77.23		100	100	99	99	100	100	100	97.03	100
	Clone 1	76.24	93.34		100	99	99	100	100	100	97.03	100
Jul	**Clone 2**	76.57	93.73	99.34		99	99	100	100	100	97.03	100
2011	**Clone 3**	76.57	93.4	98.68	99		98.02	99	99	99	96.04	99
BIW	**Clone 4**	75.91	93.1	99.97	99	98.35		99	99	99	96.04	99
	Clone 1	76.57	93.37	99.67	99.67	99	99.39		100	100	97.03	100
Aug	**Clone 2**	76.57	93.4	98.69	98.35	98.02	98.35	98.35		100	97.03	100
2011	**Clone 3**	76.57	93.73	99.67	99.67	99	99.34	100	98.35		97.03	100
BIW	**Clone 4**	75.58	93.1	98.35	98.68	98.35	98.02	98.68	97.69	98.68		97.03
	Clone 5	76.24	93.73	99.67	99.34	99	99.34	99.34	99	99.34	98.68	

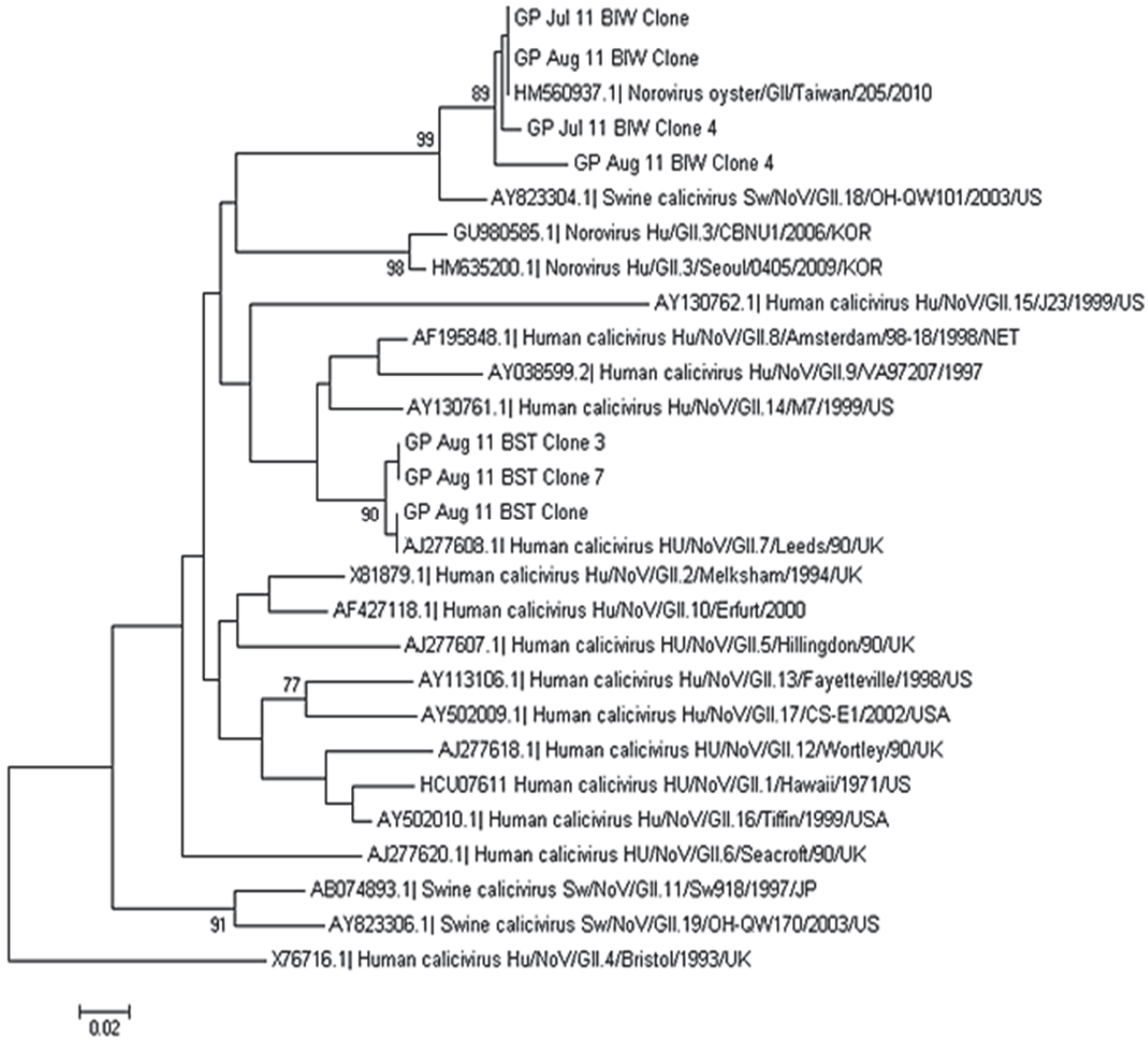


Figure 1: Neighbour-joining phylogenetic tree based on the amino acid sequence of the NoV capsid gene region. The NoV strains from irrigation water and strawberry samples along with reference NoV GII strains were used to draw the tree. Norovirus GII.4 was used to root the tree. Bootstrap percentages are indicated with the bar representing the nucleotide changes. BIW (Bon Accord Irrigation Water) and BST (Bon Accord Strawberries)

Discussion and conclusion

A wide range of NoVs have been documented to circulate in SA with NoV GII.4 being the most prevalent strain both in the surface waters and in paediatric patients in SA (Mans *et al*., 2010, 2013). Real-time RT-PCR assays in the form of multi- and singleplex assays were used to determine the presence of NoV GI, GII and HAV in paired irrigation water and strawberry samples. Noroviruses, in particularly NoV GII, were most frequently detected in the irrigation water.

Environmental sources could potentially be seen as reservoirs for animal pathogens, more specifically RNA viruses, which could potentially amount to cross-species transmission

(Heeney, 2006). The genus *Norovirus* comprises of viruses capable of infecting humans, pigs, cattle and mice species (Patel *et al*., 2009). A risk of zoonotic transmission between human and animal NoV strains could possibly exist. In such an event a novel virus with a new host tropism and increased or decreased pathogenicity may arise (Almanza *et al*., 2008). Swine NoVs are genetically and antigenically related to the most prevalent human NoVs strain (GII) (Wang *et al*., 2005). Mattison *et al*. (2007) demonstrated the presence of NoV GII.4 human-like strains in pigs whereas Wang *et al*. (2005) identified a potential human-swine recombinant NoV strain that could infect genotobiotic pigs. The possibility for pigs as potential reservoirs for the emergence of new human NoVs strains therefore exists (Wang *et al*., 2005). The contamination of fruit from irrigation water occurred in the hotter summer months when overhead irrigation was used to keep the fruit cool.

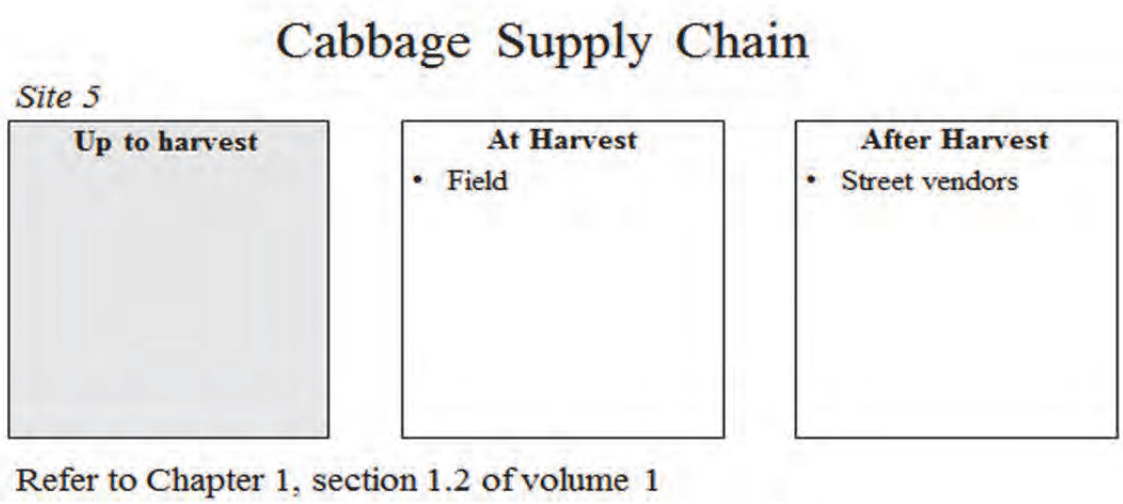
2.2.4 GAUTENG PROVINCE and KWAZULU-NATAL PROVINCE – Virological analysis of cabbages from street vendors and two farms (small-scale and commercial)

Specific aim

The objective of this study was to establish if the presence of NoVs and HAV on produce is due to contaminated irrigation water.

Experimental procedures

Sampling site



Refer to Chapter 1, section 1.2 of volume 1

Sample collection

Cabbage samples were obtained from street vendors, one farm in Gauteng and another farm in KwaZulu-Natal (KZN). To ensure that each sample was a representative sample, a sample comprised of three individual cabbages bought from the same street vendor or collected from different parts of field. The outer two leaves of the cabbages were removed and discarded and the next three consecutive leaves were sampled and analysed individually.

Viral analysis

Viral analysis including nucleic acid extraction, viral amplification and detection were as described for irrigation water and strawberries in the previous section (2.2.3).

Results

From June 2013 to September 2013 fresh produce samples (cabbage) were collected from four street vendors (4), a small-scale farm in Gauteng and another farm in KZN. No potentially pathogenic human enteric viruses, namely HAV, NoV GII and NoV GI, were detected from any of the cabbage samples (Table 11).

Table 11 Summary of results of the viral analysis of cabbage samples from four street vendors, a farm near Pretoria in Gauteng and another farm in KwaZulu-Natal

Sample	Real time RT-PCR			
	NoV GI	NoV GII	HAV	Mengovirus
Street Vendors (1-4)				
1.1	Negative	Negative	Negative	Positive
1.2	Negative	Negative	Negative	Positive
1.3	Negative	Negative	Negative	Positive
2.1	Negative	Negative	Negative	Positive
2.2	Negative	Negative	Negative	Positive
2.3	Negative	Negative	Negative	Positive
3.1	Negative	Negative	Negative	Positive
3.2	Negative	Negative	Negative	Positive
3.3	Negative	Negative	Negative	Positive
4.1	Negative	Negative	Negative	Positive
4.2	Negative	Negative	Negative	Positive
4.3	Negative	Negative	Negative	Positive
Farm 1 Gauteng				
1.1	Negative	Negative	Negative	Positive
1.2	Negative	Negative	Negative	Positive
1.3	Negative	Negative	Negative	Positive
Farm 2 KwaZulu-Natal				
1.1	Negative	Negative	Negative	Positive
1.2	Negative	Negative	Negative	Positive
1.3	Negative	Negative	Negative	Positive

Discussion and conclusion

In a previous solicited WRC project entitled "*A Quantitative investigation into the Link between Irrigation Water Quality and Food Safety*" (WRC report 1773/1-4/12, published in 2012) it was reported that Norovirus G1 was detected in one cabbage sample, however none of the viruses tested for (NoVGI, NOVGII or HAV) were detected in any of the other cabbage samples from commercial farms, subsistence farms, street vendors or commercial outlets. Similarly, in this study no enteric viruses were isolated from the cabbages tested.

2.2.5 KWAZULU-NATAL – Microbiological quality of fresh produce (broccoli, cabbage, Chinese cabbage, red cabbage, cauliflower, Crisphead lettuce, jam tomatoes, parsley and spinach) from three farms near Cato Ridge, Camperdown and Richmond respectively

Specific aim

The aim of this study was to investigate the microbiological quality of irrigation water and linked fresh produce from three farms in KwaZulu-Natal (KZN) for a period of 1 year.

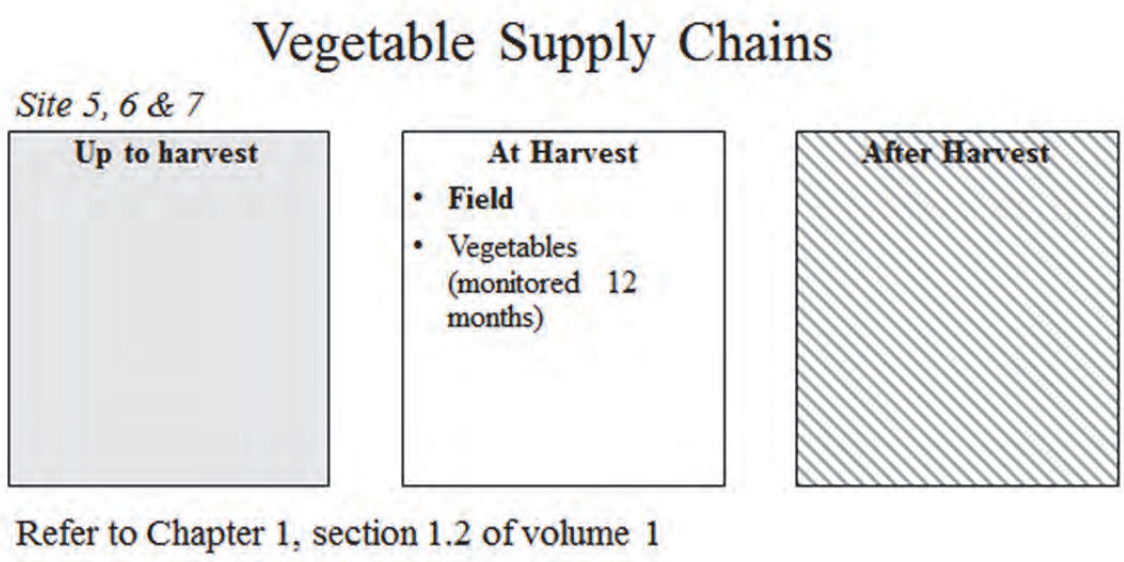
Experimental procedures

Sampling sites

Three different farms, designated A, B and C, were used in this study. Farm A (130 hectares) is located in Camperdown. Crops planted are broccoli, spinach, jam tomatoes, Crisphead lettuce, cauliflower and cabbage. Crops are not washed or packed on site. The farm has two sources of irrigation water, namely river (A1) and borehole water (A2), and the plants are irrigated weekly.

Farm B (60 hectares) is located in Cato Ridge. Crops planted are broccoli, spinach, lettuce, cauliflower, Chinese cabbage, parsley, bell pepper and red cabbage. These crops are irrigated weekly. Crops are washed and packed on site. The farm's source of irrigation water is a mixture of borehole and dam water (B).

Farm C (360 hectares) is located in Richmond. Crops planted are oranges, cabbage (planted in winter) and jam tomatoes (planted in summer). Crops are washed and packed on site. The farm's source of irrigation water is dam water (C).



Sample collection and processing

Fruit and vegetables (crops selected were determined by seasonal availability) were collected monthly from the farms for a period of 1 year from July 2009 to June 2010. Individual produce samples were prepared by adding ten grams (fresh weight) of the edible portions of vegetables/ fruit (produce sampled in triplicate) to 90 mℓ 0.1% peptone water and homogenised using a blender and serial tenfold dilutions prepared. Selective media used for growth of coliforms/*E. coli*, *Listeria monocytogenes*, *Salmonella* and *Shigella* spp. were Coliform-chromo agar, Agar Listeria Ottaviani and Agosti (ALOA) Agar respectively.

Results

Fresh produce from farm A irrigated with river water

Farm A fresh produce included broccoli, lettuce, jam tomatoes and spinach:

Coliforms – Coliform counts of broccoli, lettuce and spinach were unacceptable according to the DoH guidelines for ready-to eat fresh produce. Jam tomato coliform counts were acceptable for 50% of the samples analysed (Table 12).

E. coli – most abundant in the Crisphead lettuce (counts ranged between zero to 5.45 log CFU/g) and spinach (counts ranged between 3.7 log CFU/g to 5.9 log CFU/g). *E. coli* in the lettuce irrigated with the river water was detected 6/10 (60%) sampling times (Table 12).

L. monocytogenes – least abundant in the fresh produce tested, with the highest being recorded at 5.51 log CFU/g for Crisphead lettuce (January 2010) (Table 12).

Salmonella was detected frequently in the edible portion of the spinach and Crisphead lettuce plants (Table 12).

Campylobacter spp., coliforms and *Shigella* spp. were the most abundant on fresh produce (Table 12).

It was apparent that the fresh produce with the highest microbial contamination were the leafy vegetables, such as lettuce and spinach, throughout the season.

Table 12 The monthly variation in presumptive bacterial counts (in CFU/g) on broccoli (fields 1 and 2), Crisphead lettuce, jam tomatoes and spinach collected from farm A irrigated with river water over a one-year period

Produce/ Sampling Date	SH	SAL	LM	EC	TC	CAMP
Broccoli/field 1						
Jul-09	6.43	N	N	N	3.71	4.50
Aug-09	4.49	N	N	N	4.12	3.82
Sep-09	ND	ND	ND	ND	ND	ND
Oct-09	N	N	N	N	4.30	4.03
Nov-09 - Jun-10	ND	ND	ND	ND	ND	ND
Broccoli/field 2						
Jul-09	5.63	N	N	3.51	3.51	4.49
Aug-09	3.50	N	N	N	3.77	4.85
Sep-09	5.50	N	N	N	4.96	4.91
Oct-09	3.64	N	N	N	3.50	5.31
Nov-09 & Dec-09	ND	ND	ND	ND	ND	ND
Jan-10	3.67	N	N	N	4.31	4.54
Feb-10	ND	ND	ND	ND	ND	ND
Mar-10	3.87	3.51	N	N	4.43	4.68
Apr-10 - Jun-10	ND	ND	ND	ND	ND	ND
Crisphead lettuce						
Jul-09	5.57	N	3.61	3.70	3.82	5.66
Aug-09	3.79	N	N	N	3.62	4.26
Sep-09	3.88	N	N	N	4.84	4.62
Oct-09	N	N	N	N	4.37	4.01
Nov-09 - 7 Dec-09	ND	ND	ND	ND	ND	ND
Jan-10	5.94	3.83	4.05	5.51	5.32	4.32
Feb-10	4.47	N	N	N	4.95	4.51
Mar-10	3.98	3.55	N	5.42	5.22	4.52
Apr-10	5.83	3.67	3.93	4.47	5.24	4.33
May-10	5.73	3.71	3.83	5.45	6.09	4.29
Jun-10	5.47	3.82	N	5.37	3.99	4.44

Produce/ Sampling Date	SH	SAL	LM	EC	TC	CAMP
Jam tomatoes						
Jul-09	5.01	N	N	N	N	4.20
Aug-09	3.49	N	N	N	N	3.90
Sep-09	N	N	N	N	4.34	4.54
Oct-09 - Mar-2010	ND	ND	ND	ND	ND	ND
Apr-10	N	N	N	N	4.40	4.64
Jun-10 - May-10	N	N	N	N	4.10	4.46
Jun-10	ND	ND	ND	ND	ND	ND
Spinach						
Jul-09 - Sep-09	ND	ND	ND	ND	ND	ND
Oct-09	4.79	N	N	N	5.96	6.03
Nov-09	3.55	N	N	N	3.70	3.91
Dec-09	3.95	3.51	3.51	4.31	5.27	5.52
Jan-10	5.32	4.55	3.84	4.63	5.68	4.31
Feb-10	3.93	4.18	N	N	3.85	3.96
Mar-10	4.40	4.35	N	4.99	4.19	4.16
Apr-10	3.86	4.13	N	N	3.78	3.89
May-10	4.35	4.41	N	5.01	4.08	4.10
Jun-10	5.13	4.92	3.73	4.75	5.37	4.56

SH = *Shigella* spp.; SAL = *Salmonella* spp.; LM = *L. monocytogenes*; EC = *E. coli*; TC= Total coliforms; CAMP = *Campylobacter* spp.; ND = Not determined as the fresh produce was not present at the time of sample collection; N = bacterial species was not detected.

Fresh produce from farm A irrigated with borehole water

Farm A fresh produce included broccoli, cabbage, Crisphead lettuce, spinach and cauliflower:

Coliforms **–** Coliform counts of broccoli, lettuce and cauliflower were unacceptable according to the DoH guidelines for ready-to eat fresh produce (Table 13).

E. coli – most abundant in the Crisphead lettuce (counts ranged between zero to 5.97 log CFU/g) and was detected in 3/9 (33.33%) of the sampling times. *E. coli* was detected less frequently in the lettuce irrigated with the borehole water when compared to lettuce irrigated with the river water, which was detected in 6/10 (60%) of the sampling times.

L. monocytogenes – least abundant in the fresh produce tested, with the most being recorded at 5.97 log CFU/g in Crisphead lettuce (January 2010) which is similar to the results obtained for lettuce irrigated with river water (5.51 log presumptive *L. monocytogenes* CFU/g).

Salmonella was detected in lettuce 3/9 (33.33%) of the sampling times in the edible portion of the Crisphead lettuce plants. The microbial analysis of the fresh produce collected

from farm A irrigated with borehole water showed that the presumptive *Shigella* spp., *Campylobacter* spp. and coliform counts were found abundantly throughout the season.

Table 13 The monthly variation in presumptive bacterial counts (in CFU/g) on fresh produce irrigated with borehole water collected from farm A over a one-year period

Sampling Date	SH	SAL	LM	EC	TC	CAMP
Broccoli						
Jul-09	ND	ND	ND	ND	ND	ND
Aug-09	3.48	N	N	N	4.02	4.77
Sep -09	4.00	N	N	N	4.54	4.34
Oct-09 - Dec-09	ND	ND	ND	ND	ND	ND
Jan-10	6.18	N	N	N	4.70	4.29
Feb-09 - May-10	ND	ND	ND	ND	ND	ND
Jun-10	3.74	N	N	N	3.75	4.39
Cabbage						
Jul-09	4.54	N	N	N	N	4.19
Aug-09	5.00	N	N	N	3.72	4.81
Sep -09	4.46	4.16	N	N	4.91	6.25
Oct-09	N	N	N	4.62	N	4.92
Nov-09 - Apr-10	ND	ND	ND	ND	ND	ND
May-10	N	N	N	N	N	4.06
Jun-10	4.47	N	N	N	N	4.31
Crisphead lettuce						
Jul-09	3.96	N	N	N	N	5.83
Aug-09	4.92	N	N	N	4.47	4.85
Sep -09	4.81	N	N	N	4.54	4.86
Oct-09	4.52	N	N	N	5.91	5.84
Nov-09	N	N	N	N	4.96	3.61
Dec-09	3.49	3.54	3.50	3.56	5.56	4.68
Jan-10	5.32	4.41	3.59	5.97	5.68	4.47
Feb-10 & Mar-10	ND	ND	ND	ND	ND	ND
Apr-10	3.48	N	3.48	N	5.02	3.69
May-10	3.48	4.31	3.48	5.52	5.20	4.58
Jun-10	ND	ND	ND	ND	ND	ND
Spinach						
Jul-09	3.72	N	N	N	N	6.41
Aug-09 - Sep-09	ND	ND	ND	ND	ND	ND
Oct-09	N	N	N	N	3.85	4.08
Nov-09-Jun-10	ND	ND	ND	ND	ND	ND

Sampling Date	SH	SAL	LM	EC	TC	CAMP
Cauliflower						
Jul-09	ND	ND	ND	ND	ND	ND
Aug	5.50	N	N	N	6.05	4.97
Sep-09 - Jun-10	ND	ND	ND	ND	ND	ND

SH = *Shigella* spp.; SAL = *Salmonella* spp.; LM = *L. monocytogenes*; EC = *E. coli*; TC= Total coliforms; CAMP = *Campylobacter* spp.; ND = Not determined as the fresh produce was not present at the time of sample collection; N = bacterial species was not detected.

Fresh produce from farm B

Farm B fresh produce included: cabbage, Chinese cabbage, Red cabbage, broccoli, Crisphead lettuce, spinach and parsley.

Coliforms **–** Coliform counts of the produce were mostly were unacceptable according to the DoH guidelines for ready-to eat fresh produce (Table 14).

E. coli **–** was not detected in broccoli, cabbage, Chinese cabbage and red cabbage collected from farm B, throughout the study. In Crisphead lettuce, *E. coli* was detected at 3.54 log CFU/g during the winter period (June 2010). The *E. coli* incidence was 1/7 (14.28%) which is much lower than the lettuce from Farm A irrigated with river as well as borehole water which was 60% and 33.33% respectively.

L. monocytogenes – incidence in Red cabbage was 1/4 (25%) of the sampling times and in Crisphead lettuce 1/7 (14.28) of the sampling times. None detected in any of the other produce samples.

Salmonella was detected in spinach 4/4 (100%) of the sampling times and in Crisphead lettuce 2/7 (28.57%) of the sampling times. None were detected in the cabbage, broccoli and parley samples.

Shigella spp., *Campylobacter* spp. and coliform counts were found abundantly throughout the season.

Table 14 The monthly variation in presumptive bacterial counts (in CFU/g) on cabbage collected from farm B, over a one-year period

Produce/ Sampling Date	SH	SAL	LM	EC	TC	CAMP
Cabbage						
Jul-09 - Oct-09	ND	ND	ND	ND	ND	ND
Nov-09	3.93	N	N	N	4.63	4.67
Dec-09	4.63	N	N	N	4.73	4.05
Jan-10	4.02	N	N	N	4.91	4.34
Feb-10 - May-10	ND	ND	ND	ND	ND	ND
Jun-10	3.93	N	N	N	4.68	4.61
Chinese cabbage						
Jul-09	ND	ND	ND	ND	ND	ND
Aug-09	ND	ND	ND	ND	ND	ND
Sep-09	3.56	N	N	N	3.79	3.64
Oct-09 - Jun-10	ND	ND	ND	ND	ND	ND
Red cabbage						
Jul-09	4.92	N	3.53	N	4.06	4.20
Aug-09	3.53	N	N	N	N	3.72
Sep-09	N	N	N	N	4.00	3.71
Oct-09	N	N	N	N	4.28	4.11
Nov-09 - Jun-10	ND	ND	ND	ND	ND	ND
Broccoli						
Jul-09	N	N	N	N	N	N
Aug-09	3.50	N	N	N	5.69	6.37
Sep-09	3.87	N	N	N	3.78	4.50
Oct-09 - Feb-10	ND	ND	ND	ND	ND	ND
Mar-10	4.06	N	N	N	3.87	4.61
Apr-10 - Jun-10	ND	ND	ND	ND	ND	ND
Crisphead lettuce						
Jul-09	4.17	N	N	N	N	3.50
Aug-09	4.33	N	N	N	6.11	6.15
Sep-09	ND	ND	ND	ND	ND	ND
Oct-09	4.90	N	N	N	5.37	5.91
Nov-09	ND	ND	ND	ND	ND	ND
Dec-09	N	N	N	N	N	4.90
Jan-10 - Mar-10	ND	ND	ND	ND	ND	ND
Apr-10	N	N	N	N	N	4.81
May-10	N	3.52	N	N	N	4.76
Jun-10	4.09	3.73	3.63	3.54	4.81	5.03

Spinach						
Jul-09 - Jan-10	ND	ND	ND	ND	ND	ND
Feb-10	3.96	3.51	N	N	3.81	4.09
Mar-10	4.24	3.57	N	N	3.92	4.19
Apr-10	4.41	3.51	N	3.50	3.99	4.37
May-10	4.27	3.59	N	N	3.77	4.13
Jun-10	ND	ND	ND	ND	ND	ND
Parsley						
Jul-09 - Sep-09	ND	ND	ND	ND	ND	ND
Oct-09	4.71	N	N	4.64	4.02	4.64
Nov-09	N	N	N	N	4.30	3.83
Dec-09 - Jun-10	ND	ND	ND	ND	ND	ND

SH = *Shigella* spp.; SAL = *Salmonella* spp.; LM = *L. monocytogenes*; EC = *E. coli*; TC= Total coliforms; CAMP = *Campylobacter* spp.; ND = Not determined as the fresh produce was not present at the time of sample collection; N = bacterial species was not detected.

Fresh produce from farm C

On Farm C only cabbage was sampled over a three month period (July-September 2009).

Coliform counts of the produce were mostly unacceptable according to the DoH guidelines for ready-to eat fresh produce (Table 15).

E. coli – none detected over the sampling period.

L. monocytogenes – none detected over the sampling period.

Salmonella – none detected over the sampling period.

Campylobacter spp. and Presumptive *Shigella* spp. was found to be abundant in the fresh produce, with the highest concentration being recorded at 6.47 log CFU/g in the cabbage sample during winter (July 2009). No fresh produce samples were available for collection from farm C from March 2010-June 2010, as this farmer specialises in orange farming.

Table 15 The monthly variation in presumptive bacterial counts (CFU/g) on fresh produce collected from farm C, over a one-year period

Produce/ Sampling Date	SH	SAL	LM	EC	TC	CAMP
Cabbage						
Jul-09	6.47	N	N	N	4.81	3.79
Aug-09	N	N	N	N	5.63	6.29
Sep-09	3.95	N	N	N	N	3.51
Oct-09 - Feb-10	ND	ND	ND	ND	ND	ND

SH = *Shigella* spp.; SAL = *Salmonella* spp.; LM = *L. monocytogenes*; EC = *E. coli*; TC= Total coliforms; CAMP = *Campylobacter* spp.; ND = Not determined as the fresh produce was not present at the time of sample collection; N = bacterial species was not detected.

The results of bacterial counts on fresh produce from Farm A, B and C were summarised in Table 16.

Table 16 Range of bacterial load (lowest – highest log CFU/g) of the fresh produce over a 12-month sampling period

Fresh produce	Bacterial *spp.*					
	Coliforms	***E. coli***	***L. monocytogenes***	***Salmonella***	***Shigella spp.***	***C. jejuni***
Broccoli	3.50-4.69	0	0		0-0.43	0-6.37
Cabbage & Red cabbage & Chinese cabbage	3.64-6.25	0-4.62	0	0-4.15	0-5.00	3.64-6.25
Cauliflower	6.05	0	0	0	5.50	4.97
Crisphead lettuce	0-6.11	0-5.97	0-4.05	0-4.31	0-5.94	3.12-6.15
Jam tomatoes	0-4.40	0	0	0	3.81-5.01	3.75-4.64
Parsley	0-6. 37	0-5.01	0-3.84	0-4.92	0-5.32	3.79-6.41
Spinach	3.77-3.99	0-3.50			0-4.71	0-4.61

Discussion and conclusion

It has been reported that seasonal effects may have an impact on the survival of pathogens, with the different seasons in the year having a direct effect on the contaminated state of fresh produce by way of a change in the climatic temperature, rainfall, farming practices (such as fertiliser application) (Hall *et al*., 2002), and changes in the level of ultra-violet irradiation (Griffin *et al*., 1999). Additionally, the attachment of pathogenic bacteria to surface areas is important in their survival as they can integrate themselves into biofilms and thus, be protected from harsh conditions. The frequency of irrigation is affected by seasonal temperatures, therefore when fresh produce surfaces are in direct contact with the irrigation

water, an increase in the microbial load as well as a higher threat of contamination can be expected (Stine *et al*., 2005).

The availability of water in the soil can lead to an increase in the microbial population in plant tissues as a result of higher turgor of plants, higher plant transpiration rate and subsequent moisture accumulation on the leaf surface (Fonseca and Rushing 2006). This could explain why the highest concentrations of microbial contamination were obtained in summer, in most of the produce obtained from the different farms. High microbial counts were evident in most of the plants tested, with presumptive *Campylobacter* spp. and *Shigella* spp. being the most abundant microorganisms. However, presumptive *Campylobacter* spp. was not detected in the irrigation water of farm A (river and borehole) or B throughout the study but was evident in the fresh produce and in the soil. The leafy vegetables such as lettuce and spinach had the most microbial contamination as compared to broccoli and jam tomatoes. Crops that are grown closer to the ground are much more susceptible to contamination since they can come into direct contact with the contaminant, through splashed soil or manure, during irrigation (Hanning *et al*., 2008). Leafy vegetables with large leaf surface areas have also been shown to have a high water holding capacity and are therefore at a greater risk of contamination during irrigation (Ilic *et al*., 2009). Results obtained showed that the produce was more heavily contaminated towards maturation.

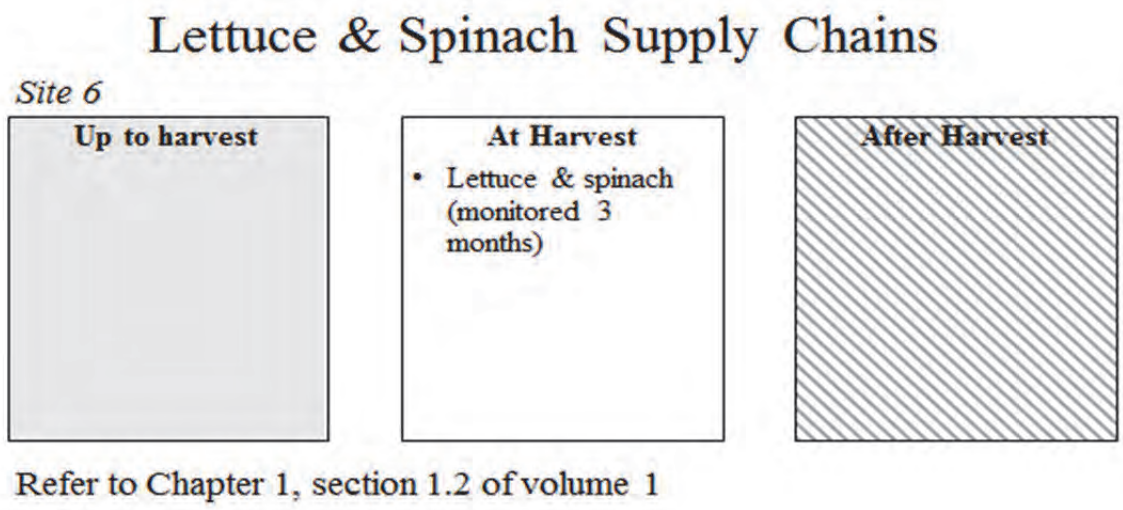
2.2.6 KWAZULU-NATAL PROVINCE – Microbiological quality and safety of leafy vegetables (spinach and lettuce) from a farm near Cato Ridge

Specific aim

The motivation of this study was to gain a broad understanding on the microbial ecology of fresh produce and how factors such as irrigation water and pesticides used during cultivation could result in contamination by foodborne pathogenic bacteria resulting in decrease in food quality and shelf life.

Experimental procedures

Sampling site



Sample collection and processing

Lettuce and spinach were sampled monthly from a farm in the Cato-Ridge area, KwaZulu-Natal over a period of 3 months (August-October 2013). The crops were irrigated twice a week. The crops were randomly collected using sterile gloves from 3 points in a cultivated field.

Ten grams of each fresh produce sample collected from the field, was used for enumeration of microbial load. The leafy fresh produce collected was washed by shaking thoroughly with 90 mℓ of 0.1% (w/v) of sterile peptone water for 30 s, allowed to rest for 30 s and shaken again for another 30 s (Gemmell and Schmidt, 2012).

Microbiological analysis

The washings obtained after shaking were used to make 10-fold dilutions which were spread plated on selective media and nutrient agar, as described in section 2.2.5. The microbial counts were expressed as CFU/g.

Results

Lettuce and spinach:

Aerobic colony counts (ACC) – the ACC counts ranged between 4.11 and 4.5 log CFU/g over the three month sampling period (Fig. 2).

E. coli – The *E. coli* counts obtained for spinach ranged from 3.3-3.7 log CFU/g and for lettuce ranged from 3.8-4.6 log during the sampling period (Fig. 3).

Presumptive *L. monocytogenes*, *Salmonella* and *Shigella* were present on lettuce and spinach and cabbage from August 2013-October 2013 (Fig. 4-6).

Combined microbiological profile of irrigation water and fresh produce:

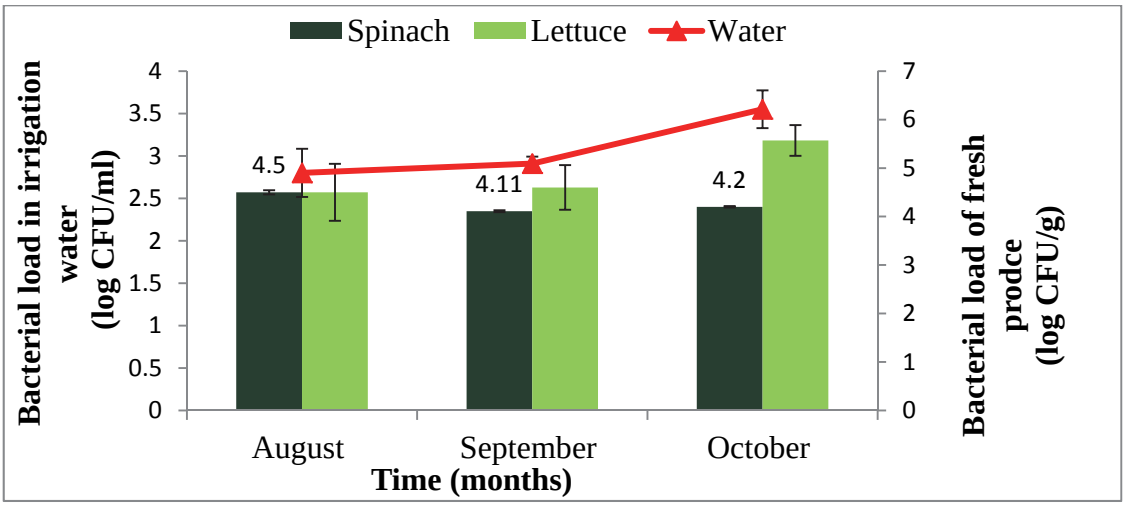


Figure 2: Average ACC detected in irrigation water and fresh produce during the sampling period

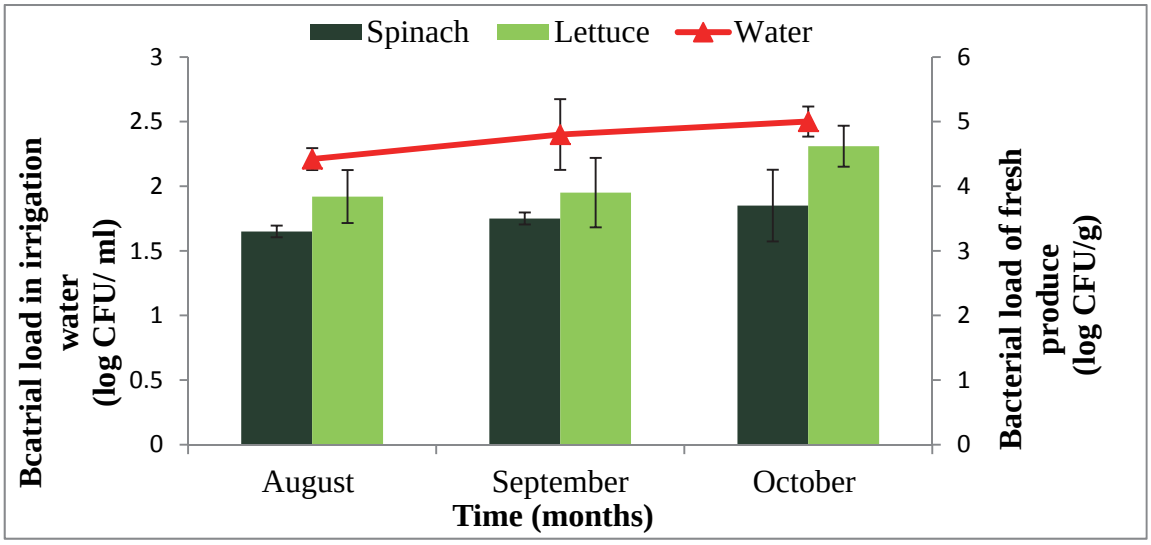


Figure 3: Average *E. coli* counts detected in irrigation water and fresh produce

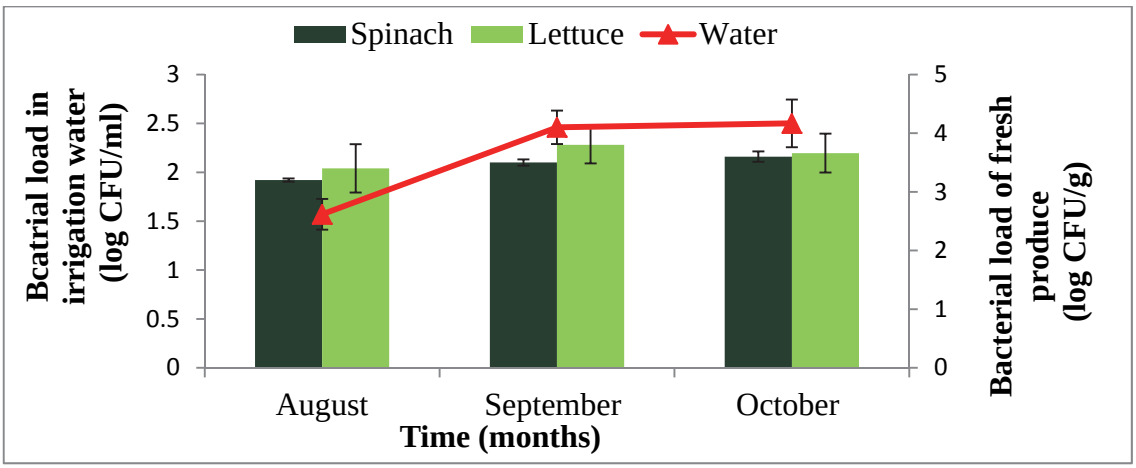


Figure 4: Average presumptive *L. monocytogenes* detected in irrigation water and fresh produce during the sampling intervals

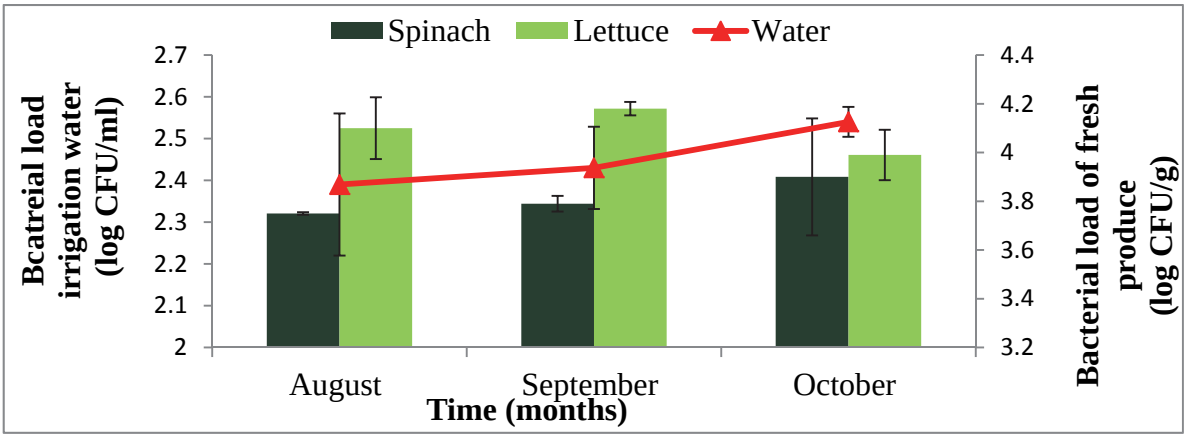


Figure 5: Average presumptive *Salmonella* counts detected in irrigation water and fresh produce during the sampling interval

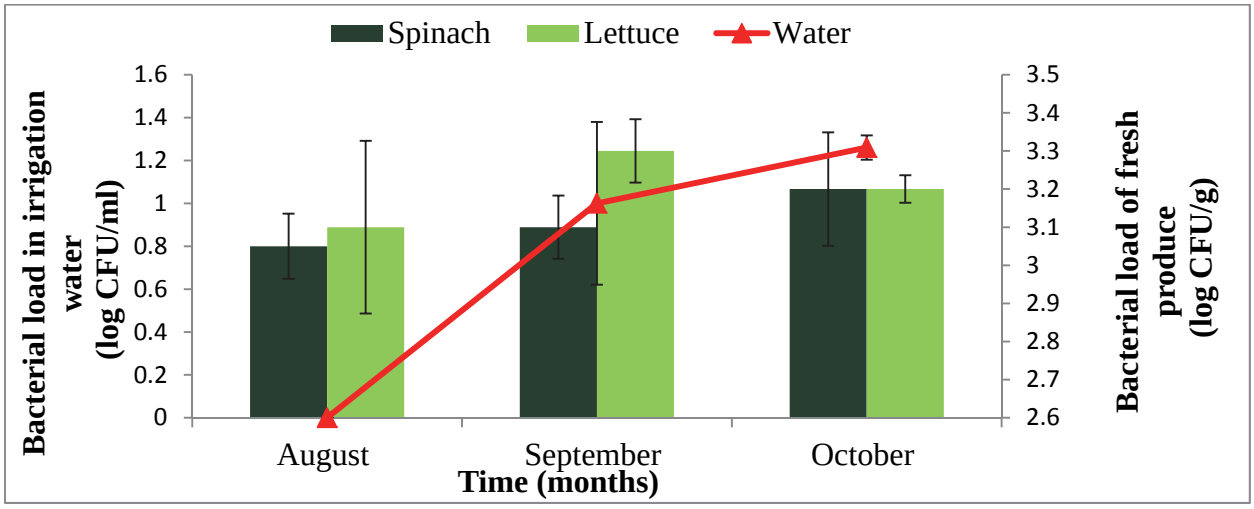


Figure 6: Average presumptive *Shigella* counts detected in irrigation water and fresh produce

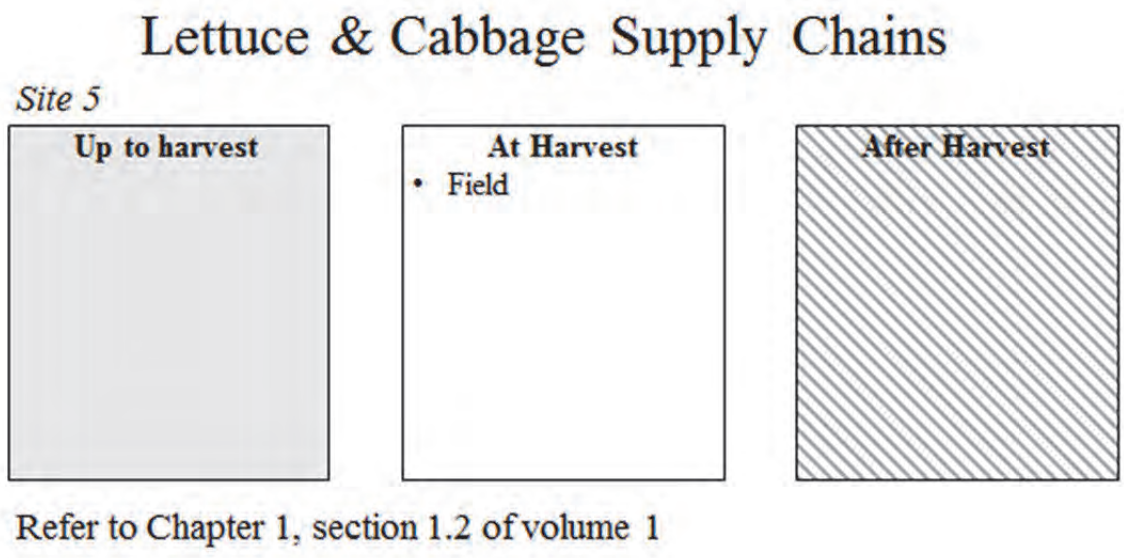
Discussion and conclusion

Similar to the results reported in section 2.2.5, the coliform counts of lettuce and spinach were > 3 log CFU/g. Additionally, the *E. coli* counts ranged between 3.3-4.6 log CFU/g, which is unacceptable according to the DoH guidelines for ready-to-eat fruit and vegetables (DoH, 2000). This could pose a potential health risk to the consumer.

2.2.7 KWAZULU-NATAL PROVINCE – Microbiological quality and safety of fresh produce (lettuce and cabbage) from two farms near Camperdown

Specific aim: To determine the microbiological quality of irrigation water, fresh produce and agricultural spray water and to show relationships among all possible pairs of physico-chemical parameters [BOD (biological oxygen demand), COD (chemical oxygen demand), total dissolved solids (TSD), turbidity and pH] and the bacterial counts found on the crops.

Experimental procedures
Sampling site



Sample collection

Lettuce (*Lactuca sativa* L.) and cabbage (*Brassica oleracea* L.) were collected randomly from three different points in the cultivated field on both farms using sterile gloves and packed into sterile re-sealable plastic bags (Tirawat *et al*., 2013). Home grown lettuce samples were also analysed and compared to the samples from the two farms. After collection, all the samples were stored on ice during transportation to the laboratory where they were stored at 4°C and processed for analysis within 24 hours (Gemmell and Schmidt, 2012).

Vegetable samples were examined for the presence of *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp., *Shigella* spp. and total heterotrophic bacteria (THB). Ten grams of leafy material was aseptically removed from each vegetable sample, weighed and placed into 90 ml 0.1% (w/v) buffered peptone water (BPW) (Merck), and agitated and rubbed by hand in sterile plastic bags for 2 min to suspend the microbes. The rinse fluids obtained were used to determine the bacterial counts. Ten-fold serial dilutions of the water and vegetable samples were prepared from which 100 µl was spread plated onto selective media (viz. Chromocult Coliform Agar (Merck) for the selection of *E. coli*, Listeria mono Diffrential Agar (Base) with supplements (Fluka) for *L. monocytogenes* and *Salmonella-Shigella* Agar (SSA) (Merck) for *Salmonella* and *Shigella* spp. and Plate Count Agar (PCA) (Merck) for THB). The plates were inverted and incubated at 37ºC for 24-48 hours. All microbial counts were reported as CFU/g for the vegetables and CFU/ml for the water samples (Temiz *et al*., 2011).

Confirmation of presumptive pathogen identities

DNA isolation

Genomic DNA isolation was done using the boiling method according to Ahmed *et al*., (2014).

PCR amplification and confirmation of isolates

PCR amplification of DNA from *L. monocytogenes* was performed according to Jeyaletchumi *et al*. (2010). For the amplification of the *mdh* gene in *E. coli*, the protocol used by Roohollah and Mahjar (2013) was used. For the amplification of the *InvA* gene in *Salmonella* spp., the protocol by Gassama-Sow *et al*. (2006) was used. Details of primers used for PCR analysis were summarised in Table 17.

Table 17 The description of the primers, sequences and target genes.

Organism	Target gene	Primers	Primer sequences ⟶	Product size (bp)
E. coli	*mdh*	*mdh*-F	CGTTCTGTTCAAATGGCCTCAGG	392
		mdh-R	ACTGAAAGGCAAACAGCCAAG	
L. monocytogenes	*hlyA*	*hlyA*-F	CAAGTCCTAAGACGCCAATC	1412
		hlyA-R	ATAAAGTGTAGTGCCCCAGA	
Salmonella spp.	*InvA*	*InvA*-F	TGCCTACAAGCATGAAATGG	450
		InvA-R	AAACTGGACCACGGTTGACAA	

Statistical analysis

Data of the log averages and associated standard deviations (SD) from each set of three replicates were calculated for each sample time using Excel 2010 (Microsoft). The data was statistically analysed using mean and standard deviations. P-values associated with T-test and One-way ANOVA was taken as an indicator of significant differences among the data using GraphPad Prism software (version 5.00). In all statistical analyses, confidence level was held at 95% and $P<0.05$ (at 5% level of significance) was considered as significant. Pearson product-moment correlation coefficient using SPSS (SPSS, Inc., Illinois 21.0) was used to show relationships among all possible pairs of physico-chemical parameters and the bacterial counts found on the crops.

Results

Lettuce and cabbage:

Total Heterotrophic bacteria (THB) – HTB counts ranged from 3.88-7.21 and 3.08-6.74 log CFU/g for lettuce and cabbage, respectively (Fig.7). The means were found to be significant ($p \leq 0.05$) for irrigation water and vegetables on farm A, and highly significant ($p < 0.01$) for irrigation water and vegetables on farm B. Lettuce generally had a higher microbial load than cabbage.

E. coli counts on cabbage ranged between 0.52-1.12 log CFU/g and on lettuce between zero and 3.2 log CFU/g (Fig. 8).

Presumptive *L. monocytogenes* and *Salmonella* were present on some of the lettuce and cabbage samples (Fig. 8).

Microbiological profile of irrigation water, potable water and fresh produce was summarised in Fig. 7.

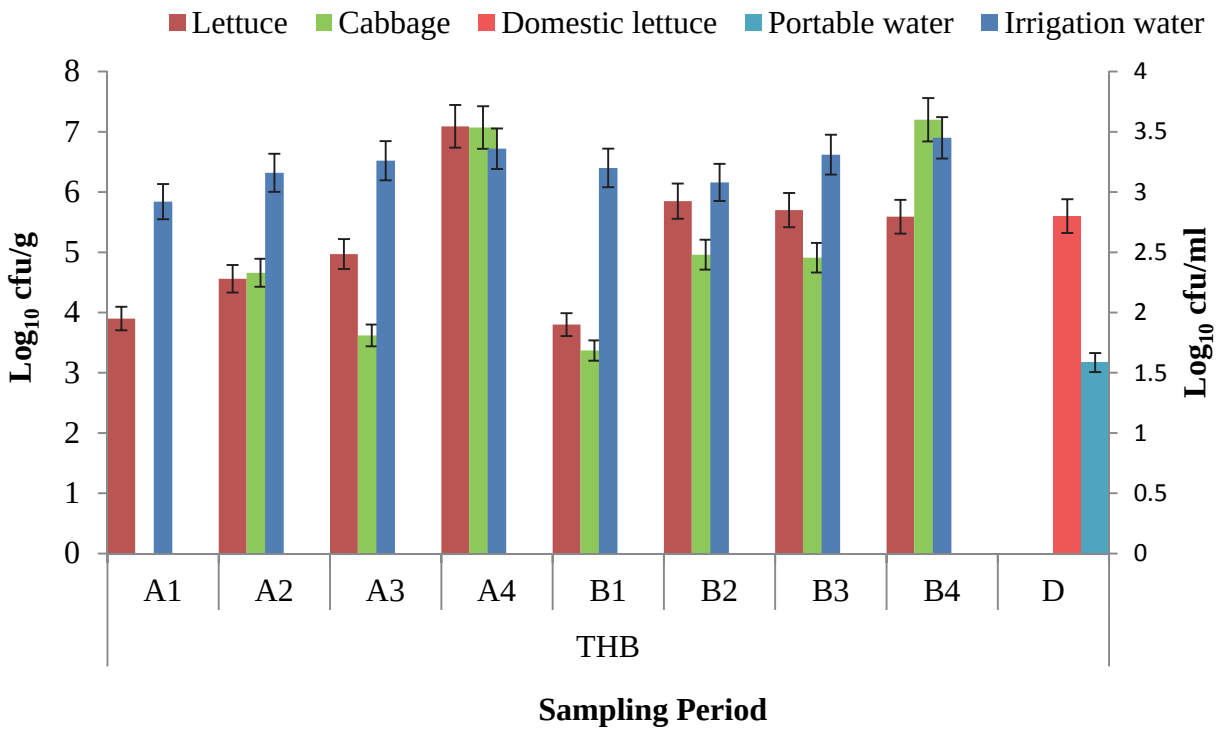


Figure 7: Microbial load of total heterotrophic bacteria in irrigation water and fresh produce.

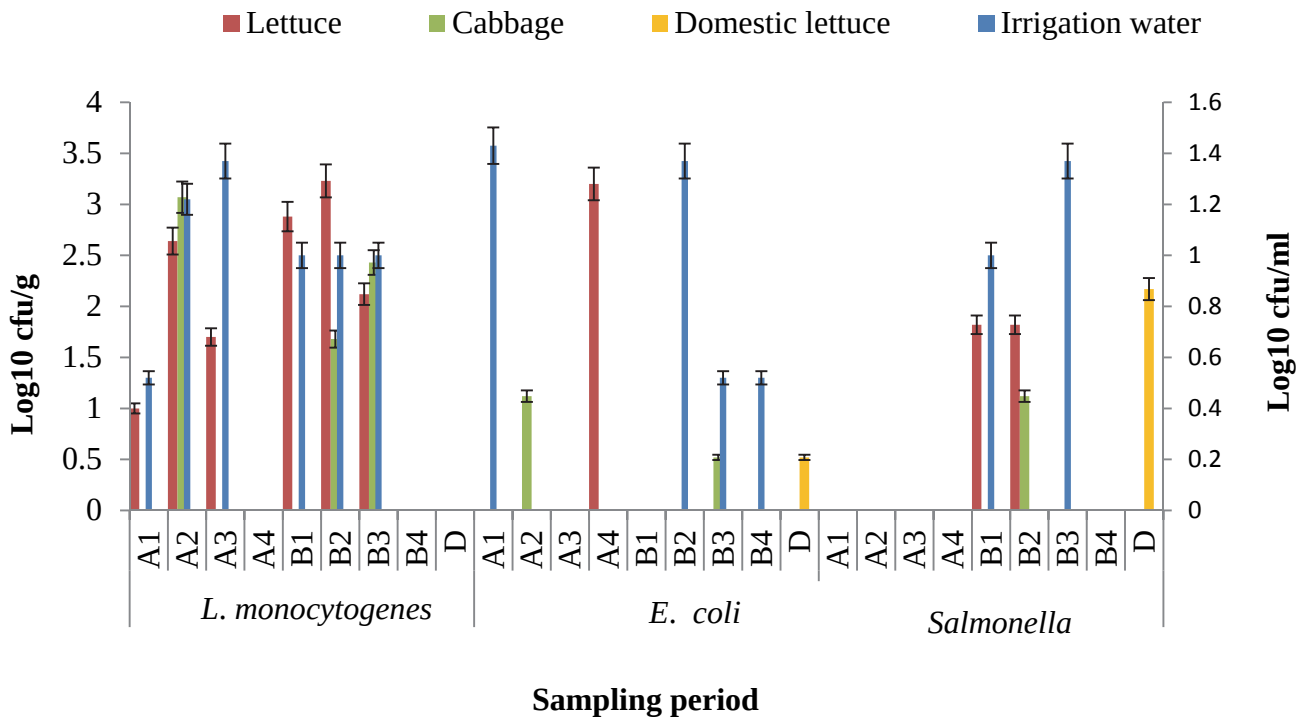


Figure 8: Microbial load of selected bacteria in irrigation water and fresh produce.

Discussion and conclusion

The microbial load on lettuce was generally higher compared to cabbage samples. Slight differences in the micro-architectural structure of the vegetable may also contribute to differences in pathogen attachment and survival. Similarly, higher numbers of *Salmonella* spp. were found on Romaine lettuce compared to cabbage surfaces (Monaghan and Hutchison, 2012). Lettuce irrigated with non-potable water has been shown to have significantly higher rate of total coliforms and presumptive *Salmonella* contamination than lettuce irrigated with drinking water (Strawn *et al*., 2013). Chai *et al*., (2011) showed that *Salmonella* spp. present on the surface of plants can survive for extended periods and might overcome the immune response of plants and actively enter through the stomata. This was demonstrated for lettuce. No presumptive *Shigella* spp. was detected in any of the samples analysed. *Shigella* is not ubiquitous in the environment and contamination of fresh produce by this species has been mostly linked to improper sanitation and human handling (Nygren *et al*., 2013).

2.2.8 LIMPOPO PROVINCE – Microbiological quality and safety of onions and tomatoes from a commercial farm near Polokwane

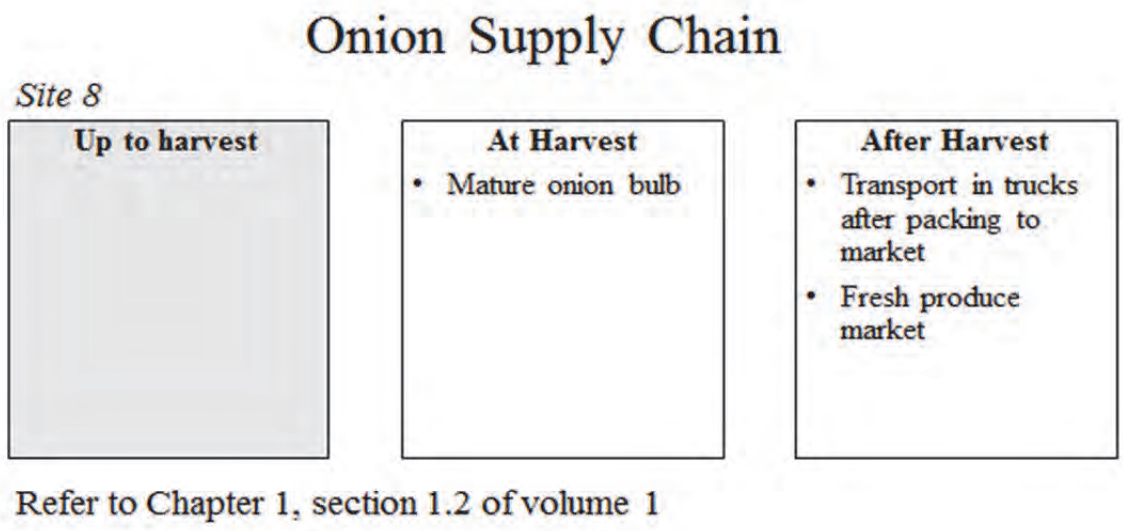
Specific aim

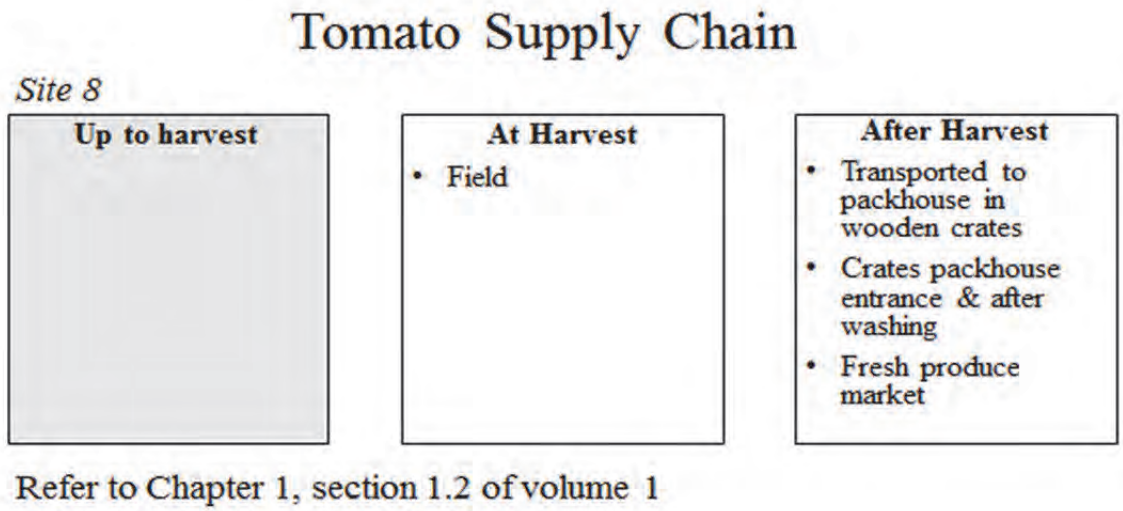
The aim of this project was to investigate the quality of irrigation water and onions in the field “up to” and “at” harvest, after drying in the middens in the field and at the fresh produce market.

Experimental procedures

Experimental site

A commercial onion/tomato production farm situated near Polokwane in the Limpopo Province. Irrigation water was pumped from the Sand River to holding dams and used for overhead irrigation of the onions. The tomatoes are grown in open fields. Irrigation water is pumped from the Sand River into a catchment dam and pumped through black PVC pipes into the field where tomatoes are irrigated using drip-irrigation. Tomatoes are harvested, transported to the packhouse, washed with Sporekill-treated water and hand-packed into boxes. The tomatoes are transported to the Tshwane Fresh Produce Market in non-refrigerated trucks, where it is sold to the retail and hospitality industries the next day.





Onion sampling and processing

Onion replicate samples (comprised of three sub-samples per replicate) were systematically sampled from four rows in the onion field at different growth stages, i.e. 3-4 leaves, 5-7 leaves and 2-3 cm bulb size (before harvest), at harvest and after harvest (from the fresh produce market). The onions were sampled at the different growth stages to determine at which stage/s potential human pathogenic bacteria (*E. coli* (including O157:H7), *Salmonella* spp. and *Listeria monocytogenes*), present in the irrigation water, were detected in onions. The harvested whole onions were placed in labelled bags and then transported to the laboratory as described for the water samples. The outer dry leaves of the mature onions were removed, the onions chopped up, using a sterile knife and cutting board. Three twenty-five grams sub samples of each replicate were weighed into a sterile polyethylene bag containing 225 mℓ of

0.1% peptone buffered water (BPW) (Merck, Johannesburg, South Africa) and macerated for 5 min at 230 rev min-1 in a Seward Stomacher 400 Circulator (Seward Ltd., London, UK).

Tomato sampling and processing

Samples were collected at harvest in the field from plants at three different locations in the same block. At the packhouse, tomatoes were sampled from wooden crates at the entrance and after washing. Specific boxes were marked on the day of packing. Tomatoes from the marked boxes were sampled at the fresh produce market the following day. All samples were taken in triplicate and five tomatoes represented a single sample.

Each tomato sample was weighed, litre Ringer's solution supplemented with Tween-80 added and sonicated in an ultrasonic bath for 5 min. The fruit was removed and the solution vacuum filtered through a 45 µℓ membrane. Each tomato sample was washed in an ultrasonic bath in one litre Ringer's solution supplemented with Tween-80. The fruit was removed and the solution vacuum filtered through a 0.45 µM filter membrane.

The washed tomatoes were then halved and a half of each tomato blended to a purée with an electric blender. Twenty-five grams of purée were transferred to stomacher bags containing 225 mℓ PBW. The bags were stomached for 2 minutes.

Microbiological analysis

Each 0.45 µM filter membrane was cut into small pieces, added to 9 mℓ TSB medium and serially diluted. Bacteria, fungi and yeasts on tomato surfaces were numerated as described in Section 1, microbiological analysis.

The stomached tomato (25 g) samples were enriched by incubation at 37°C for 24 hours to facilitate detection of human pathogenic bacteria.

Confirmation of microorganism identities using MALDI-TOF MS

Coliform colonies were isolated from 3M Petrifilm media (3M), streaked onto EMB agar to obtain single colonies and incubated for 24h at 37°C. The identities of the 24 h isolates were determined using Matrix-assisted laser desorption ionization time-of flight mass spectrometry (MALDI-TOF) as described in Section 2.2.1 for parsley.

Molecular methods

DNA was extracted from single colonies of presumptive *E. coli*, *Salmonella* and *Listeria* isolates using the Quick-gDNA miniprep kit (Zymo Research, California, USA). The universal primers F-27 and R-1492 were used to amplify the 16s rDNA genes of the bacterial isolates (Lane *et al*., 1985). The PCR products were sequenced and a search of nucleotide data bases conducted to confirm the microorganism identities.

Antibiotic resistance of *E. coli* isolates

Confirmed *E. coli* isolates from water and fresh produce were subjected to antibiotic susceptibility testing using the Kirby Bauer disk diffusion technique (Bauer *et al*., 1966). Confirmed *E. coli* isolates were cultured in Brain Heart Infusion Broth at 37°C for 24 hours and hundred microlitres of the bacterial suspension were plated onto Mueller-Hinton agar plates (Merck). The antibiotic agents and concentrations used are summarised in Table 18 (Mast Diagnostics, UK, supplied by Davies Diagnostics, Midrand, SA). Antibiotic resistance

or susceptibility of *E. coli* isolates were scored according to criteria developed by the National Committee on Clinical Laboratory Standards (CLSI 2007).

Table 18 Summary of antibiotic agents and concentrations used in this study

Class	Antibiotic	Abbreviation	Concentration
Penicillins	Augmentin (Amoxicillin / Clavulanic Acid)	AUG30C	20 µg / 10 µg
	Ampicillin	AP10C	10 µg
	Aztreonam	ATM30C	30 µg
	Cefepime	CPM30C	30 µg
	Cefotaxime	CTX30C	30 µg
	Cefquinome	CEQ30C	30 µg
	Ceftazidime	CAZ30C	30 µg
Cephems	Ceftriaxone	CRO30C	30 µg
Phenicols	Chloramphenicol	C30C	30 µg
Quinolones	Enrofloxacin	ENF5C	5 µg
Quinolones	Nalidixic Acid	NA30C	30 µg
	Florphenicol	FFC30C	30 µg
Tetracyclines	Tetracycline	T30C	30 µg
Folate Pathway Inhibitors	Cotrimoxazole (Trimethoprim/Sulfamethoxazole)	TS25C	1.25 µg / 23.75 µg

Results

Microbiological analysis of onions

Aerobic colony counts (ACC) – The maximum ACC count on the onion surfaces was 6.9 CFU/g (3-4 leaf stage) and 2 log CFU/g at the fresh produce market (Fig. 9). The onion microbial load gradually declined during the onion production cycle up to marketing, where it was comparable to microbial specification guidelines for fresh fruit and vegetables in South Africa.

Coliforms and E. coli – The total aerobic bacteria and coliform counts decreased significantly from the 3-4 leaf stage to the 2-3 cm bulb stage and the counts for the market onions were not statistically different to the values measured at the 2-3 cm bulb stage (Fig. 9).

No *E. coli* was counted for any of the onion samples using coliform/*E. coli* count plates. In contrast, *E. coli* was isolated from 10% of the onion samples, but only after selective enrichment of the onion samples indicating its presence at levels lower than the standard detectable limit for plate counts.

Fungi/ Yeasts – The yeast and fungi counts were very low ranging from 0.77 log CFU/g (lowest) to 1.82 log CFU/g (highest) at the early growth stages, where after the yeast/fungi counts were zero (results not shown).

E. coli O157H7 – None of the human pathogenic bacteria tested for were isolated from onions at any of the growth stages throughout production and marketing.

Salmonella spp. – None isolated.

Listeria spp. – None isolated

Dominant microorganisms on onion surfaces included *E. cloacae, Pantoea ananatis, Pseudomonas chlororaphis, P. koreensis, Fusarium oxysporum, Penicillium* spp., *Aspergillus niger, Microsphaeropsis arundis* and *Alternaria alternata.*

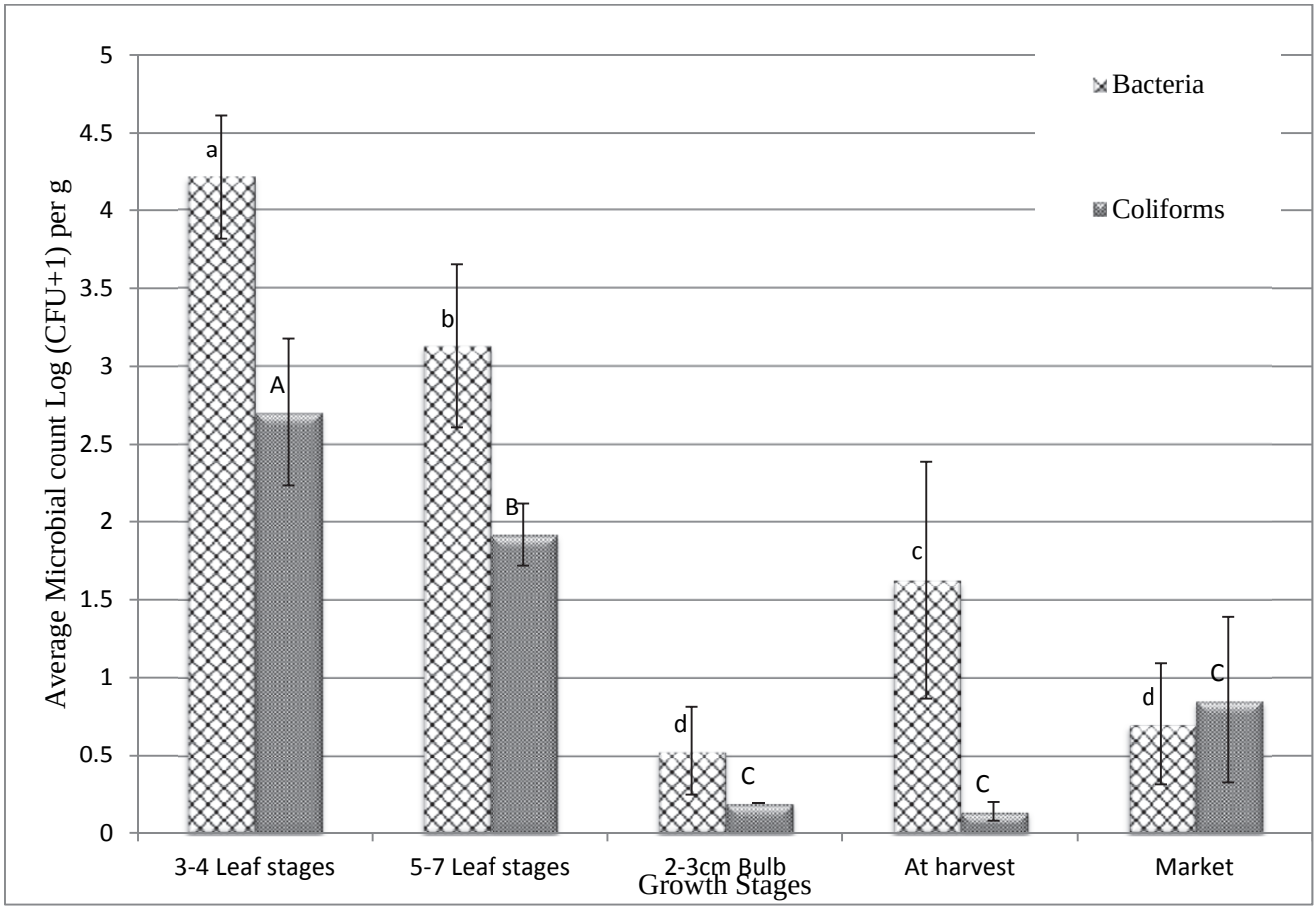


Figure 9: Total aerobic bacteria and coliform counts measured for onions sampled at the different growth stages. *The E. coli* counts of the onions were zero for all the sampling times.

Microbiological analysis of tomatoes

Aerobic colony counts (ACC) – The maximum ACC count was 3.8 log CFU/g for tomatoes after washing in the packhouse and the minimum count 1.8 log CFU/g for tomatoes sampled at the fresh produce market (Fig. 10).

Fungi and yeast counts – fungal and yeast numbers were low, i.e. < or = to 1 log CFU/g = 10 CFU/g (Fig. 10).

E. coli O157H7 and *S.* Typhimurium – None of the human pathogenic bacteria tested were isolated from tomatoes.

Microorganisms isolated from tomatoes included *Citrobacter freundii, Klebsiella pneumonia, Acinetobacter* genomo species 3, *Serratia marcescens, Enterobacter cloacae, E. aerogenes, E. ludwigii, E. asburiae, Pseudomonas fulva, P. putida, P. stutzeri,* and *P. mendocina.*

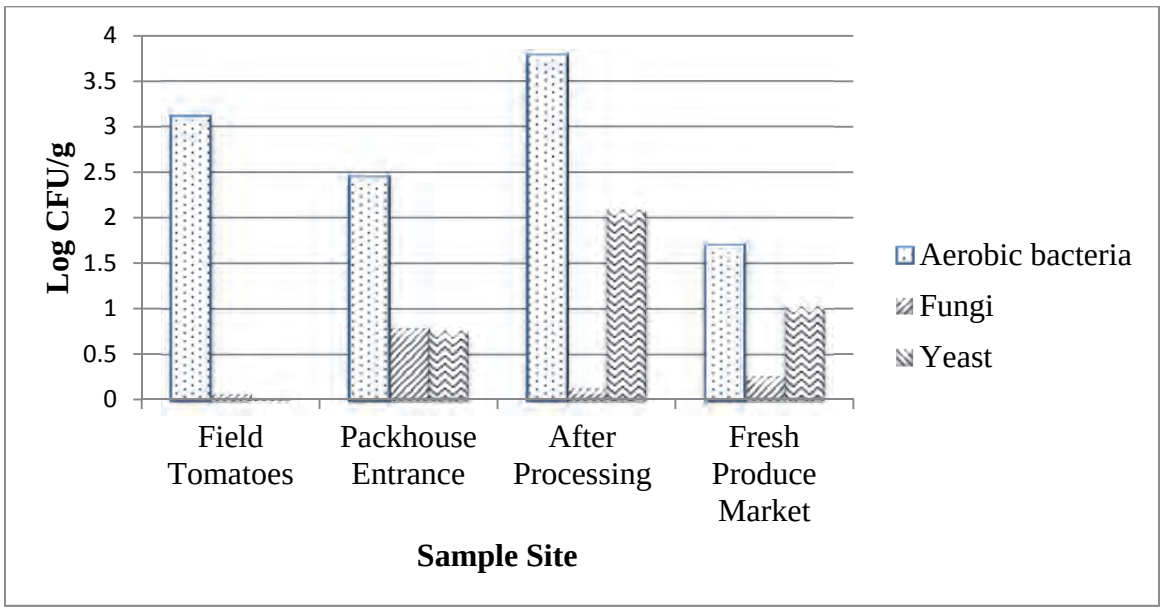


Figure 10 Graph showing the total number of aerobic bacteria, fungi and yeast on the tomatoes.

Escherichia coli (including O157:H7), *Salmonella* and *Listeria monocytogenes* prevalence in onion and tomato samples

Selective media used for identification of presumptive *E. coli*, *Salmonella* and *Listeria monocytogenes* after enrichment in appropriate media were not always efficient, since many false positive results were obtained. Presumptive *E. coli* colonies isolated from EMB agar after selective enrichment were identified as either *E. coli*, *Enterococcus hirae*, *Enterobacter cloacae*, *Enterobacter absuriae* and *C. freundii* using MALDI-TOF analysis and 16s ribosomal DNA amplification, sequencing and blast analysis. Results obtained using 16s ribosomal DNA PCR analysis of the colonies (results not shown) showed that 97% of the environmental isolate identities determined using MALDI-TOF analysis were correct. Typical colonies of *E. coli* O157:H7 were not detected in any of the onion samples after streaking onto Agar Chrom ID TM O157H7 (BioMerieux, Marcyl-l' Etoile, France) or PCR analysis using the *uidA* gene primers targeting the mutated *uidA* gene of *E. coli* O157:H7 as described by Cebula *et al*. (1995).

As was observed for *E. coli* identification, the chromogenic media (XLD and SBM) used for identifying presumptive *Salmonella* isolates were not always efficient. MALDI-TOF analysis identifies *Salmonella* only at the genus and not at the species level. MALDI-TOF analysis of presumptive *Salmonella* isolates from the selective media showed that they were false positives. These results were confirmed using 16s ribosomal PCR analysis.
All presumptive positive *Listeria monocytogenes* colonies isolated from enriched onion samples by streaking onto the Listeria chromogenic media were identified as either *Bacillus* spp., *Enterobacter* spp. or *Staphylococcus* spp. using MALDI-TOF analysis.

Antibiotic resistance of environmental *E. coli* isolates from the onions

Although only five *E. coli* strains were isolated from the onions the antibiotic resistance profiles included resistance against one or more of the following antibiotics: penicillins (Ampicillan & Aztreonam), phenicols (Chloramphenicol), quinolones (Enrofloxacin & Nalidixic acid)), Tertacyclines (Tetracycline) and folate pathway inhibitors (Trimethoprim Sulfametoxazole) (Table 19).

Table 19 Summary of results obtained for antimicrobial resistance of environmental *E. coli* isolates

		No. (%) of resistant isolates
Antimicrobial(s)	**Amt(s)/disc (µg)**	**Onion samples (n=5)**
AP 10C	10 µg	2 (40.0)
AUG 30C	20 µg / 10 µg	0 (0)
ATM 30C	30 µg	2 (40.0)
CPM 30C	30 µg	0 (0)
CTX 30C	30 µg	0 (0)
CEQ 30C	30 µg	0 (0)
CAZ 30C	30 µg	0 (0)
CRO 30C	30 µg	0 (0)
C 30C	30 µg	1 (20.0)
TS 25C	1.25 µg / 23.75 µg	1 (20.0)
ENF 5C	5 µg	1 (20.0)
FFC 30C	30 µg	0 (0)
NA 30C	30 µg	1 (20.0)
T 30C	30 µg	1 (20.0)

Virulence gene analysis of environmental *Escherichia coli* isolates from the onions

One of the onion *E. coli* isolates (n=5) harboured the *stx1* gene.

Discussion and conclusion

The decrease observed in the number of total aerobic bacteria, coliforms, fungi and yeasts on the onion surfaces over time during onion production from growth to marketing could possibly be ascribed to 1) known antimicrobial properties of onions against Gram-negative and Gram-positive microorganisms (Hughes *et al*., 1991), and 2) the drying out of the onions in the middens in the field. The reduction in microorganism numbers on onions observed in this study is in agreement with results of another study where populations of *Salmonella* and *E. coli* O157:H7 inoculated with 10^7 CFU/9cm^2 on onions skins, showed a decline of 3 log CFU/g after drying for 30 min (Lieberman *et al.,* 2013).

E. coli was not isolated from any of the onion and tomato samples using coliform/*E. coli* count plates, however *E. coli* was isolated after selective enrichment of the onion samples indicating its presence at levels lower than the standard detectable limit for plate counts.

In this study the aerobic bacterial counts for the tomatoes varied between 1.8 log CFU/g to 3.8 log CFU/g and *Salmonella* was not detected in any of the tomato samples. Similarly, Gómez *et al.* (2009) reported the mesophilic bacterial counts were lower than 4 log CFU/g on tomatoes. Total aerobic levels of 2 log CFU/g were reported for tomatoes (Cardenas *et al.*, 2013).

Traditionally classical and genomic identification methods were used to identify micro-organisms, however MALDI-TOF MS has been reported to be an alternative method for taxonomical applications (Böhme *et al*., 2011; Dieckmann and von Dohren, 2005). When compared to conventional 16S rRNA gene sequencing, similar results were obtained with MALDI-TOF MS analysis and enabled identification to the subspecies level (De Bruyne *et al*., 2011; Dieckmann and Malorney, 2011). However, to date MALDI-TOF MS has predominantly been used to identify clinical isolates (Ferreira *et al*., 2011; Kroumova *et al*., 2011). A limited number of studies have reported the application of MALDI-TOF MS in a microbial ecological context. The diversity and distribution of cultivable bacteria throughout the whole spinach process line, including the fresh produce and the wash water, was determined (Hausdorf *et al.*, 2013). In a study to evaluate the microbiological quality of the Msunduzi River in KwaZulu-Natal, South Africa, the identities of *E. coli* isolates were confirmed using PCR and MALDI-TOF MS (Gemmel and Schmidt, 2012). The identification results using the two methods correlated, as was observed in this study. The results of this study confirmed the suitability of MALDI-TOF MS as an alternative method for identification of human pathogenic bacteria isolated from water and fresh produce, comparable to results obtained with PCR analysis.

2.2.9 LIMPOPO PROVINCE – Microbiological quality and safety of peaches from a commercial peach farm near Mookgophong

Specific aim

The aim of this study was to investigate the microbiological quality of peaches and water used for irrigation purposes with the ultimate aim of establishing whether a link between the irrigation water and fresh fruit can be made.

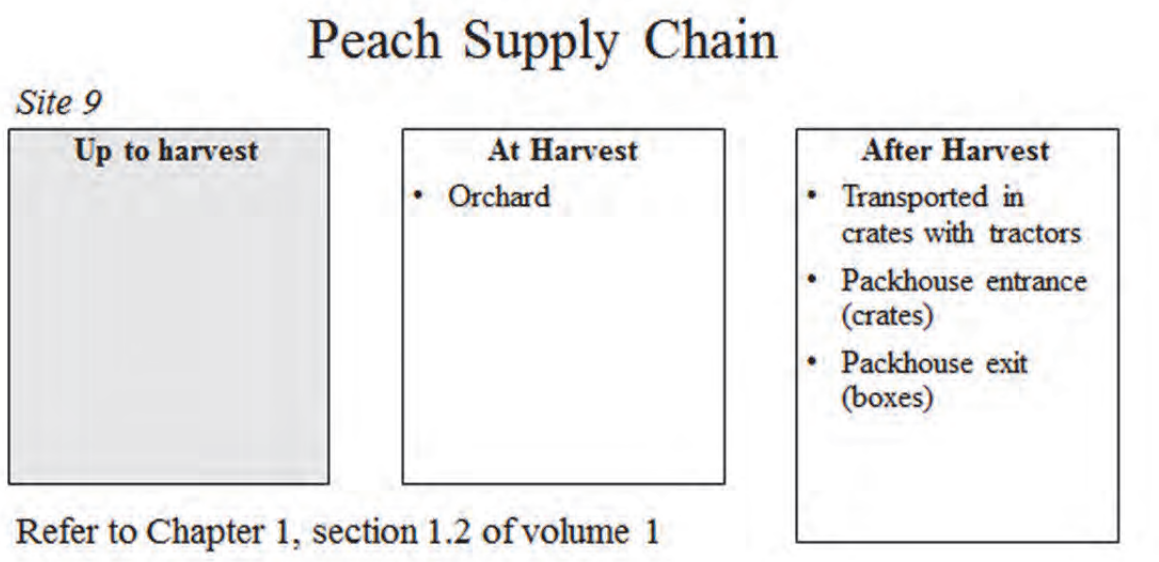
Experimental procedures

Experimental site

- The study was conducted on a Global GAP (Good Agricultural Practices) certified commercial farm. Peaches (Cultivar=Sunette) were sampled from a peach farm located in the Mookgophong (Naboomspruit) area in the Northern Province, South Africa.
- The peach farm is Global GAP (Good Agricultural Practices) certified and has a traceability system in place.
- Water from the Sterk River and dams in the river is used for irrigation.
- Peaches are irrigated by use of micro irrigation and pivots.
- For the sake of our study, a single orchard (HH) on the farm was selected for microbiological assessment of peaches.

Process flow

After harvesting in the orchard, the peaches are packed into plastic crates and transported on a tractor to the packhouse. Upon arrival at the packhouse, the peaches are sorted according to size using a size selector line and packed by hand where after it is dispatched in cooled trucks to the fresh produce market in Johannesburg.



Peach sampling and processing

From the orchard five batches of eight fruit each were sampled from the crates on tractors arriving at the packhouse. Five batches of eight fruit each were sampled from the boxes after packing. For all samples, microbiological analysis was conducted within 24 hours after sampling.

Sonication

Each batch of peaches were transferred into a beaker containing 1liter Ringers' solution with 0.02% Tween 80, the displacement volume noted, and sonicated in a Digital Ultrasonic Cleaner (EUmax®, Labotec, Model no. UD200SH-6L) for 30 seconds. After removal of the peaches, the solution was filtered, using 0.45µm filter membranes.

Microbiological analysis

Total aerobic colony counts, coliform/*E. coli*, fungi and yeast counts were determined as described for onions (section 2.2.8). Slanetz and Bartley medium (Oxoid) supplemented with 2,3,5-triphenyltetrazolium chloride (TTC solution, Oxoid) and Staph Express count system (3M Petrifilm) were used for the enumeration of Intestinal enterococci (IE) and *S. aureus* respectively. Colonies were enumerated after incubation of plates at 37°C for 24-48 hours. Presumptive intestinal enterococci colonies were transferred onto Bile Esculin Azide Agar (Oxoid) and incubated at 44°C for 2 hours (ISO 7899-2:2000). Enrichment for the detection of *Escherichia coli* O157: H7, *Salmonella* Typhimurium and *Listeria monocytogenes* were done as described in section C1. Identities of single colonies of presumptive positive colonies

isolated from chromogenic media were confirmed using MALDI-TOF analysis (see section 2.3.1).

Results

Peaches

Aerobic colony counts (ACC) – The results showed that the aerobic plate counts for the peach surfaces from the orchard, crates entering the packhouse and in boxes after packing were similar ranging between 4 and 4.5 log CFU cm^2 (Table 20).

Coliforms and E. coli – Coliform counts were similar ranging from 1.6 log CFU/ cm^2 in the orchard to 1.3 log CFU/ cm^2 in boxes after packing (Table 20). *E. coli* was isolated from orchard samples only at < 1 log CFU/ cm^2.

Enterococci – counts of 0.22 log CFU/ cm^2 on the surfaces of peaches from the orchard were detected and none on peaches in the crates entering the packhouse or after packing (Table 20).

Yeasts and fungi – Yeast numbers were similar to the total aerobic bacterial counts ranging between 4 to 4.5 log CFU/ cm^2 (Table 20). Fungi were detected on peaches from the orchard at 0.7 log CFU/ cm^2 and not on packhouse peaches.

E. coli O157 (including H7) **–** none isolated.

Salmonella – none isolated.

Listeria spp. – none isolated.

The dominant bacteria identified on the peach surfaces were *Pantoea agglomerans, Bacillus cereus, Enterobacter cowanii* and *Enterococcus hirae*. Dominant fungi isolated from MEA medium were identified as *Fusarium* spp., *Alternaria* spp. and *Epicoccum nigrum.*

Table 20 Summary of results obtained for the microbiological analysis of peaches

Produce, sites	**Average microbial counts log CFU/ cm^2**				
Peaches	**Aerobic bacteria (ACC)**	**Fungi**	**Yeast**	**Petrifilm**	**Enterococci**
Orchard	3.80 (0.19)	1.81 (0.09)	3.91 (0.2)	1.38 (0.17)	0.31 (0.02)
Crates Entrance)	4.16 (0.21)	0.00	4.00 (0.2)	1.19 (0.06)	0.43 (0.02)
Packhouse Exit	3.55 (0.18)	0.00	3.74 (0.19)	0.99 (0.05)	1.01 (0.05)

Discussion and conclusion

The microbiological quality of the peaches was acceptable according to DoH guidelines for ready-to-eat fruit and vegetables. *E. coli* was detected on orchard peaches only, *E. coli* O157 (including H7), *Salmonella* spp. and *L. monocytogenes* were not detected in any of the fruit samples from the orchard, at the packhouse entrance or exit after packing. The peaches can generally be regarded as acceptable for human consumption based on these criteria.

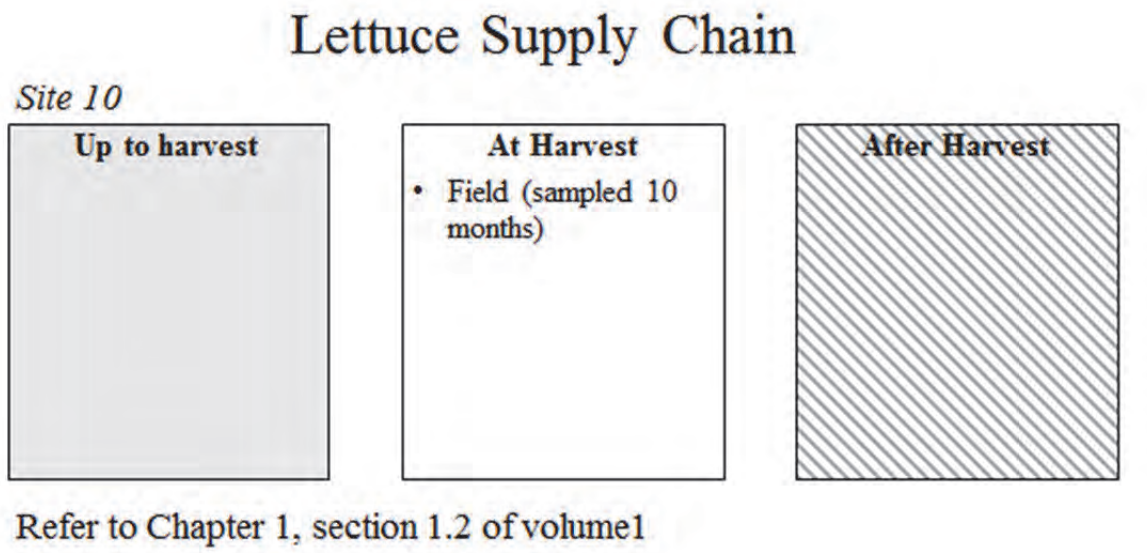
2.2.10 NORTH WEST PROVINCE – Microbiological quality and safety of lettuce from a farm near Skeerpoort

Specific aim: The aim of this study was to determine whether irrigation water from the Skeerpoort River located in the North West Province of South Africa was a source of foodborne bacterial pathogens on irrigated lettuce over two seasons (10 months).

Experimental procedures

Site Selection

A vegetable-growing farm using water from a dam, containing water diverted from the Skeerpoort River for irrigation purposes, was chosen as the sampling site for lettuce. The Skeerpoort River is downstream of the Hartbeespoort Dam, a major tourist location surrounded by an urban settlement.



Sample Collection

Lettuce: Crisphead lettuce samples were collected monthly from a field irrigated with water from the Skeerpoort River for 10 months from January to October 2011.

Microbiological analysis

Bacterial indicators (Aerobic colony counts, ACC; Anaerobic spore formers, AnSF; Aerobic spore formers, ASF; Intestinal enterococci, IE; Total coliforms, TC; Faecal coliforms, FC) and pathogens (*E. coli*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella* spp.) were enumerated and identified (Table 21).

Characterization and identification of aerobic bacteria

For each lettuce sample analysed, 3 colonies were randomly picked from the highest dilution standard plate-count agar petri-dish. Gram stain and catalase tests were done. Thirty (30) bacterial isolates were used. 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, Oklahoma, USA, 2011) to test for significant differences in microbiological quality at 95% confidence interval between sampling intervals.

Table 21 Methodology for detection of bacterial organisms on lettuce

Organism	Media	Temperature/ Time of incubation	Company	Reference
Aerobic colony count (ACC)	Standard Plate Count agar	30°C for 48 to 72h	(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, 1998)
Anaerobic spore formers (AnSF)	Trypticase Soy Agar	35°C for 48 h		
Total coliforms	Brilliant Green Bile 2% Broth (BGLB)	35°C for 24 to 48h	(Oxoid Ltd, Basingstoke Hampshire, UK)	MFHB19 (Health Canada, 2002)
Faecal coliforms	EC Broth			
E. coli	Eosin Methylene Blue Agar (Levine) (EMB)	35°C for 24h		
	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)	
Intestinal enterococci	Slanetz and Bartley Medium	35°C for 44h	(Oxoid Ltd, Basingstoke Hampshire, UK)	(SANS ISO 7899-2) (2004)
	Bile Esculin Agar	44°C for 2h		
S. aureus	Baird-Parker Agar	35°C for 48h	(Oxoid Ltd, Basingstoke Hampshire, UK)	SABS ISO 6888-1 (1999)
§*Salmonella* spp	Brilliance ™ Salmonella Agar Base	35°C for 24 to 48h	(Oxoid Ltd, Basingstoke Hampshire, UK)	SABS ISO 6579 (2003)
	Rapid Salmonella	35°C for 24 to 48h	BioRad	
†*L. monocytogenes*	Listeria Selective Agar (Oxford formulation)	35°C for 48 h	(Oxoid Ltd, Basingstoke Hampshire, UK)	SABS ISO 11290-1 (1996)
	Palcam Selective Supplement			

§-Final confirmation of presumptive *Salmonella* spp. was by serotyping at the Agricultural Research Council (ARC)-Onderstepoort, Pretoria, South Africa. **†**-Final confirmation of presumptive *L. monocytogenes* was done using Omnilog® Data Collection Software Identification System Version 2.1 (Biolog Inc. Hayward, California)

Antimicrobial testing

Ten isolates from lettuce irrigated with water from the Skeerpoort River were used in the study. Antibiotics used included: Amikacin (30 µg), Gentamicin (10 µg), Chloramphenicol (30 µg), Nalidixic Acid (30 µg), Norfloxacin (10 µg), Neomycin (30µg), Nitrofurantoin (300 µg), Amoxicillin (25 µg), Ampicillin (10 µg), Cephalothin (30 µg) and Oxytetracycline (30 µg). Antibiotic susceptibility testing was determined by disc diffusion method using Mueller-Hinton agar (Oxoid).

Virulence gene testing

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, Hercules, California). 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).

Results

Lettuce:

Aerobic colony counts (ACC) – The maximum ACC noted was 7.3 log CFU/g during August and the lowest during June and July, i.e. 4.9 and 5.1 log CFU/g respectively (Fig. 11). There was a significant difference ($p \leq 0.05$) in ACC over the different months.

Anaerobic spore formers (AnSFs) – the maximum count was 5.5 log CFU/g and the minimum 1 log CFU/g (Fig. 11).

Aerobic spore formers (ASF) – the counts ranged between maximum 4.3 log CFU/g and minimum 3 log CFU/g and were present at all the sampling intervals (Fig. 11).

S. aureus – the counts ranged between 3.9 log CFU/g (maximum) and 1.5 log CFU/g (minimum and were present during 9 of the 10 sampling intervals (Fig. 11).

Faecal coliforms (FC) – FC counts varied between 2.8 log CFU/g and 3.5 log CFU/g were present during 5 of the 10 intervals (Fig. 11).

E. coli **–** *E. coli* was present during 3 of 10 sampling intervals (Fig. 11).

E. coli O157:H7 – absent

Salmonella spp. – absent

L. monocytogenes – present

Microorganism identification

Prevalence of predominant bacteria from lettuce irrigated with water from the Skeerpoort River for isolates (n=30) collected monthly over 10 months were summarised in Table 22.

Irrigation water and lettuce: The microbial counts and percentage of water and lettuce samples (n=20) positive for pathogens for samples collected between January and November 2011 from the Skeerpoort River and adjacent lettuce field were summarised in Table 23.

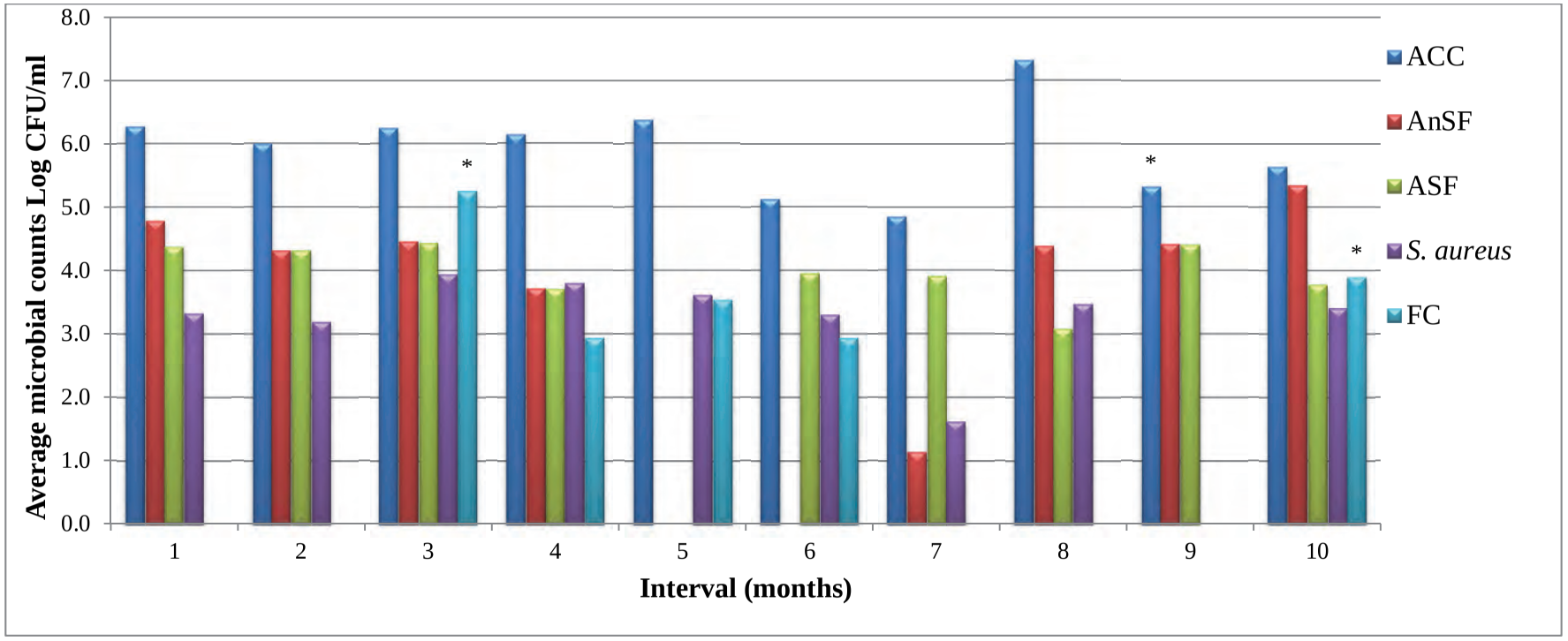


Figure 11: Change in bacterial counts and incidence of *E. coli* on lettuce irrigated with water from the Skeerpoort River for samples (n=10) collected monthly over 10 months. (*) interval positive for *E. coli*. Aerobic colony count (ACC), Anaerobic spore formers (AnSF), Aerobic spore formers (ASF), *Staphylococcus aureus* (*S. aureus*) and Faecal coliforms (FC).

Table 22 Prevalence of predominant bacteria from lettuce irrigated with water from the Skeerpoort River for isolates (n=30) collected monthly over 10 months

Month	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Total number of isolates**
Percentage isolates											
E. coli	–	–	33	–	67	67	33	–	100	67	22
Bacillus spp	67	33	–	33	33	–	33	100	–	–	17
Enterobacter spp	–	33	33	–	–	–	–	–	–	–	7
Klebsiella spp	–	–	33	–	–	–	–	–	–	–	3
Enterococcus spp	–	–	–	–	–	–	33	–	–	–	1
Salmonella spp	–	–	–	–	–	–	–	–	–	–	1
Serratia spp	–	–	–	–	–	–	–	–	–	33	1
Raoultella spp	–	–	–	–	–	33	–	–	–	–	2
Leclerciaa decarboxylata	33	–	–	–	–	–	–	–	–	–	1
Brevibacillus borstelensis	–	–	–	33	–	–	–	–	–	–	1
Exiguobacter iumundae	–	–	–	33	–	–	–	–	–	–	1
Staphylococcus kloosi	–	33	–	–	–	–	–	–	–	–	1
Bautiaxella agrestis	–	–	–	–	–	–	–	–	–	–	1
Burkholderia caprophylil	–	–	–	–	–	–	–	–	–	–	1
Total number of isolates	3	3	3	3	3	3	3	3	3	3	60
ACC (log CFU/mℓ)	6.3	6.0	6.3	6.1	6.4	5.1	4.9	7.3	5.3	5.6	

– Not determined ACC – aerobic colony counts.

Table 23 Summary of microbial counts and percentage of water and lettuce samples (n=20) positive for pathogens for samples collected between January and November 2011 from the Skeerpoort River and adjacent lettuce field.

Sample	No. of samples	Indicator parameters				% of samples positive for pathogens			
		Aerobic Colony Count (ACC)	Anaerobic spore formers AnSF	Aerobic spore formers ASF	Faecal coliforms	*Salmonella* ssp.	*Listeria* ssp.	*E. coli*	Other Microorganisms
		Log CFU/mℓ			Log CFU/100mℓ				
Skeerpoort River water[1]	10	3.4±0.7 [a]	2.5± 1.8 [a]	2.5±1.2 [a]	1.3 ±1.1	0	0	40	70
Lettuce [2]	10	5.9±0.7 [b]	3.3±2.0 [b]	3.6±1.9 [b]	1.9±2.1	0	30	30	100
Average	20	4.7±1.5	2.9± 1.9	3.1±1.4	1.6±0.4	0	15	35	85

Antibiotic resistance in *E. coli* isolated from the Skeerpoort River and irrigated lettuce

E. coli from lettuce irrigated with water from the Skeerpoort river were resistant to antibiotics (amoxycillin, ampicillin, cephalothin and oxytetracycline) (Table 24). 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 24).

Table 24 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	**Lettuce (n=10)**
Amikacin, 30 µg	–
Gentamicin, 10 µg	–
Chloramphenicol, 30 µg	–
Nalidixic acid, 30 µg	–
Norfloxacin, 10 µg	–
Neomycin, 30 µg	–
Nitroforantoin, 300 µg	–
Amoxycillin, 25 µg	10.0
Ampicillin, 10 µg	50.0
Cephalothin, 30 µg	60.0
Oxytetracyline, 30 µg	20.0

\- No resistance

Resistance of isolates to more than one antibiotic was 50% in both the Skeerpoort River and 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 24).

Three antibiotic resistance patterns were noted among isolates from lettuce (Table 25). More isolates from the Skeerpoort River showed resistance to multiple antibiotics (Table 25).

Table 25 Multiple resistances to antibiotics in *E. coli* collected over 10 months from the Skeerpoort River and lettuce irrigated with water from the Skeerpoort 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	-
KF-AMP-NA	8.3	-
KF-AMP-AML	8.3	-
KF-AMP-OT	5.3	-
KF-OT-NA-AMP	8.3	-
AMP-AML	-	10.0
AMP-OT	-	20.0

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

Discussion and conclusion

The high Aerobic colony counts (ACC) on lettuce is probably due to the fact that lettuce grows in close proximity to soil which contains high levels of bacteria. Additionally, fresh vegetables have large numbers of bacteria as part of their normal flora (Gilbert *et al*., 2000). In recent studies the ACC in irrigated fresh vegetables was reported to range from 4.3 to 10.4 log CFU/g (Halablab *et al*., 2011) and 3 to 4 log CFU/g (Ijabadeniyi, 2010). Ijabadeniyi (2010) reported low counts of aerobic and anaerobic spores on broccoli and cauliflower irrigated with water from the Loskop canal.

The presence of *L. monocytogene*s on lettuce indicates contamination either from the water source or the soil it is cultivated in. Fertilization of soil with manure has been shown to increase the risk of contaminating produce with *Listeria* spp. because of its high concentrations (Ackermann, 2010). *Listeria monocytogenes* present in soil containing manure can be transferred onto vegetables where it can survive for an extended time period. Presence of *L. monocytogenes* on lettuce is of concern because it was associated with a foodborne outbreak associated with cantaloupes in the United States leading to a recall of produce and fatalities (Centers for Disease Control and Prevention (CDC) 2011).

The ability of *E. coli* to proliferate on lettuce could have been enhanced by the large surface area of its leaves and foliar surfaces with many folds and fissures, which provided good shelter for the pathogen (Halabalabet *et al*., 2011). Additionally bruises and cuts on vegetables could have been possible points of attachment for bacterial pathogens because slight abrasions on leaves from processes such as dropping irrigation water can cause microscopic damage, which could facilitate pathogen proliferation (Aruscavage, 2007). Furthermore, moisture and exudates from plant material and cuts leach nutrients through bruises that promote microbial growth (Ampadu, 2007).

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). High multiple resistances to antibiotics within *E. coli* isolated from the Skeerpoort River may be linked to existence of a large pool of antibiotic resistant bacteria.

Rivers such as the Skeerpoort River, flowing through urban areas, may have increased exposure to higher levels of pollution attributed to domestic and industrial waste (Walters *et al*., 2011). This increases the risk of contamination with antibiotic resistant bacterial pathogens that may be transferred on to irrigated produce such as lettuce. 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 can serve as reservoirs of antibiotic resistant pathogens posing a food safety and public health threat. 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). 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. Additionally similar genetic relatedness shown by some isolates from irrigation water and lettuce further implicates irrigation water as a source of pathogenic bacterial contamination.

Results from this study show that surface irrigation water sources in South Africa can be reservoirs of antibiotic resistant *E. coli*. Additionally irrigation water with poor bacteriological quality may contaminate irrigated produce, presenting a risk to both food safety and public health.

2.2.11 NORTH WEST – Microbiological quality and safety of Crisp lettuce from harvesting to end of shelf life in the Brits area

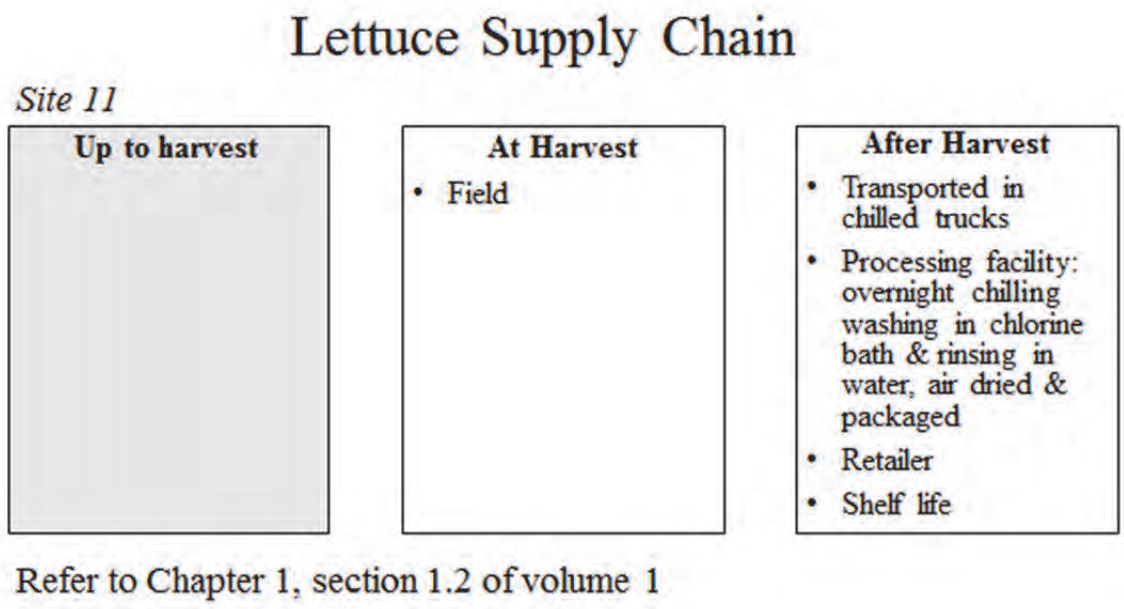
Specific aim

The objectives of this study were to evaluate the microbiological quality of irrigation water and field lettuce from planting to end of shelf life. Additionally the antimicrobial susceptibility of selected intestinal enterococci isolated from field-lettuce samples were determined.

Experimental procedures

Experimental site

- The lettuce farm is located in the Brits area in the North West province, South Africa.
- The lettuce farm is Global GAP (Good Agricultural Practices) certified and has a traceability system in place.
- Water from the Crocodile River and Hartbeespoort Dam feeds the irrigation canal channelled to the farm for irrigation.
- Lettuce plants were irrigated by use of overhead sprinklers.
- For the sake of our study, a single plot on the lettuce farm was selected for microbiological assessment.



Process flow

After harvesting, the lettuce heads were packed into unlined plastic crates (six heads per crate), stored temporarily in a chilled packing house and transported in a chilled truck at 5°C to the processing factory about 60 km from the farm. Upon receiving at the factory, the crates were chilled overnight at 5°C in a high humidity refrigerator for processing the following day. After overnight chilling, the external leaves and the lettuce core were removed, and the lettuce leaves were shredded using knives and cutting boards. The leaves were conveyed into an agitated water bath containing 200 ppm chlorine water at pH 4.8, rinsed in another water bath, dried by centrifugation after entering the high care area and packaged in 150 g packs. The processing factory had documented Hazard Analysis and Critical Control Points (HACCP) and was in the process of acquiring HACCP certification.

Sampling and sample preparation

On the farm, irrigation water samples were taken once every two weeks during week 1 (a day after planting), weeks 3, 5, 7 and 10 when the mature lettuce heads were harvested. All samples were taken sequentially by following the same crop through growing, processing, packaging and storage. On the farm, samples were taken once every two weeks on week 1

(one day after planting), weeks 3, 5, 7 and 10 when the mature lettuce heads were harvested. Six lettuce samples and irrigation water samples were taken on the farm. In the processing factory, 6 lettuce samples were taken at each sampling point. Lettuce packs from the processing factory were stored in the laboratory at 5°C for 8 days to simulate retail shelf life. During the storage period, 6 lettuce packs were sampled on each of days 2, 4, 6 and 8 for microbiological analyses. Additionally, three swabs of food contact surfaces were also taken from the farm during harvesting of lettuce and at the processing factory during lettuce processing. Water and lettuce samples were processed as described for irrigation water and lettuce in the previous section (2.2.10).

Microbiological analysis

Total aerobic colony counts (ACC), Intestinal enterococci counts (IE), total coliform counts (TC), *E. coli* counts and *Staphylococcus aureus* counts were determined as described in the experimental procedures for lettuce in the previous section (2.2.10). Typical *E. coli* isolates were further assessed for the presence of Shiga Toxin producing *E. coli* (STEC: stx1, stx2, ehx, eae and O157:H7) and enterohaemorrhagic *E. coli* serotypes (O145, H7, O26, O111 and O103) by real time PCR using gene discs (GeneSystems, France). *Salmonella* spp. and *Listeria* spp. were presence/absence were as described in the previous section.

Results

The bacterial incidence in water and on lettuce from planting to end of shelf life were summarised in Table 26.

Lettuce:

Microbiological quality of lettuce and food contact surfaces in the processing plant

The changes in populations of ACC, total coliforms, *S. aureus* and intestinal enterococci (IE) on lettuce sampled at various points along the processing line are illustrated in Figure 12.

Aerobic colony counts (ACC) – ACC for field lettuce samples ranged between 7.2 log CFU/g (week 1) and 6 log CFU/g (week 10). Washing in the packhouse reduced ACC to 3.6 log CFU/g.

Total coliforms – the maximum count was 6.4 log CFU/g and washing in the packhouse reduced the coliform counts with approximately 3 log CFU/g.

Intestinal enterococci (IC) – present at low numbers in the field (ranging from zero CFU/g to a maximum of 2.5 log CFU/g). Not present after washing in the packhouse and throughout the shelf life study.

E. coli – present in the field lettuce samples, however no *E. coli* observed at reception in packhouse, after chilling, washing and throughout storage at 5C°C for 8 days.

Salmonella spp. – *Salmonella* spp. was detected in lettuce packs on day 8 of storage only.

Listeria spp. – present in field samples, at reception and after overnight chilling, absent after washing and throughout the shelf life study.

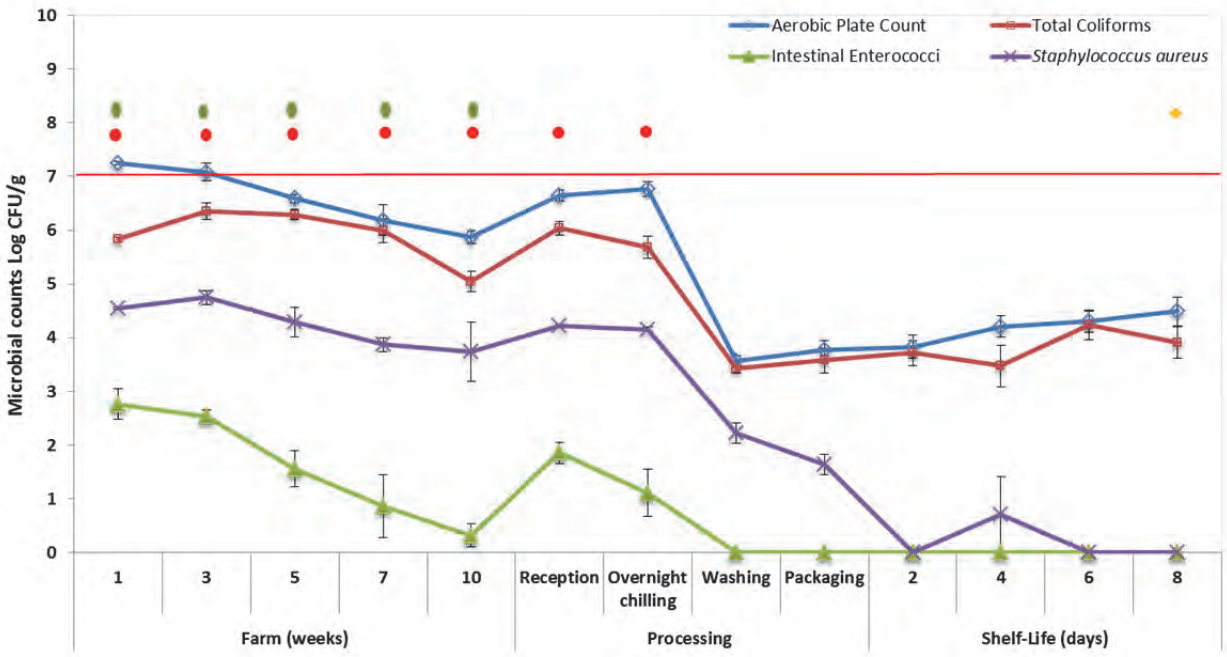


Figure 12: Changes in bacteria concentrations/populations on crisp lettuce from planting to end of shelf life ◆*Salmonella* spp; ● *Listeria* spp; ⬮ *Escherichia coli*

Table 26 Summary of bacterial incidence on lettuce from planting to end of shelf life.

		Log_{10} CFU/g or mℓ					% Incidence	
Sampling points	**N**	**Aerobic Plate Count**	**Total Coliforms**	**Intestinal Enterococci**	***S. aureus***	***E. coli***	***Listeria***	***Salmonella***
Soil	24	6.6 (0.34)	5.7 (0.44)	2.0 (0.93)	4.6 (0.39)	25 (6)	87.5 (21)	ND
Lettuce:								
Farm	30	6.6 (0.64)	5.9 (0.60)	1.6 (1.24)	4.2 (0.76)	10 (3)	63 (19)	ND
Processing factory	30	5.3 (1.44)	4.8 (1.15)	0.7 (0.91)	3.3 (1.21)	ND	10 (3)	ND
Shelf life storage	30	4.1 (0.56)	3.8 (0.71)	0 (0)	0.5 (0.76)	ND	ND	10 (3)

ND: Not Detected

The bacteria isolated from irrigation water, soil and lettuce along the entire supply chain are summarised in Table 27.

Table 27 Bacteria isolated from irrigation water, soil and lettuce along the entire supply chain

Organism type	Isolates	Pathogenic status	Irrigation water	Soil	Lettuce (farm)	Lettuce at receiving	During processing	Shelf-life
Pathogens	*E. coli*							
	E. coli O157:H7[a]	NP						
	Salmonella	NP						
Listeria	*Listeria ivanovii*	P						
	Listeria welshimeri	NP						
	Listeria grayi	NP						
	Listeria innocua	NP						
Intestinal Enterococci	*Enterococcus faecium*	OP						
	Enterococcus faecalis	OP						
	Enterococcus avium	NP						
	Aerococcus viridans	NP						
	Globicatella saguinis	NP						
Coliforms	*Acinetobacter baylyi*	OP						
	Aeromonas caviae	OP						
	Agrobacterium tumefaciens	NP						
	Enterobacter cloacae	OP						
	Enterococcus gallinarum							
	Enterobacter cowanii	U						
	Pantoea agglomerans	P						
	Pseudomonas rhodesia	NP						
	Pseudomonas cichorii	NP						
	Pseudomonas koreensis							
	Pseudomonas oryzihabitants	OP						
	Pseudomonas monteilii	OP						
	Raoultella ornithinolytica	OP						
	Stenotrophomonas sp.							

Pathogen status: P (Pathogenic), NP (Non-pathogenic), OP (Opportunistic pathogen), U (Unknown)

[a]*E. coli* O157:H7 did not have the toxin genes stx1, stx2, eae and ehx characteristic of Shiga Toxin producing *E. coli*. Shaded columns indicate positive samples for the respective bacterial isolates

Antibiotic resistance profiles for *Enterococcus faecium* and *Enterococcus faecalis* isolated from lettuce field samples are summarised in Table 28.

Table 28 Antibiotic resistance profiles for *Enterococcus faecium* and *Enterococcus faecalis* isolated from lettuce field samples

Antibiotic	*Enterococcus faecalis* (N = 4)	*Enterococcus faecium* (N = 9)
	% Resistance	
Amoxicillin (10 µg)	0 (0)	11 (1)
Ampicillin (10 µg)	0 (0)	0 (0)
Erythromycin (15 µg)	0 (0)	11 (1)
Nitrofurantoin (300 µg)	75 (3)	67 (6)
Tetracycline (30 µg)	25 (1)	22 (2)
Vancomycin (30 µg)	75 (3)	11 (1)
Gentamicin (10 µg)	100 (4)	22 (2)
Ciprofcoxacin (10 µg)	100 (4)	56 (5)

The correlation of total coliform counts with the incidence of pathogens on lettuce are summarised in Table 29.

Table 29 Correlation of total coliform counts with the incidence of pathogens on lettuce.

Total coliforms	N	% Positive samples		
		Escherichia coli	*Listeria spp.*	*Salmonella spp.*
<100	0	0	0	0
100-1000	5	0	0	0
1000-10000	19	0	0	5 (1)
>10000	60	5 (3)	37 (22)	3 (2)

Discussion and conclusion

Any effective and pragmatic approach to mitigating risks of microbiological contamination in the lettuce supply chain requires the identification and understanding of contamination sources and management strategies to reduce risks. Our study showed that aerobic bacteria, coliforms, intestinal enterococci, *S. aureus, E. coli, Listeria* and *Salmonella* occurred on lettuce. In this study, the increase in aerobic colony counts, total coliform counts and intestinal enterococci on lettuce heads between harvesting and reception at the processing factory suggest the possibility of temperature abuse if harvested lettuce heads were left to stand at ambient temperatures for extended periods prior to chilling. However, further increase after overnight chilling indicates the presence of mesophilic microorganisms that can multiply under chilling conditions. Washing and rinsing in chlorine water at 200ppm reduced the aerobic colony counts by 3 log CFU/mℓ. Similarly, Ruiz-Cruz *et al*. (2007) demonstrated a 2 to 3 log CFU/g reduction of *L. monocytogenes, Salmonella* and *E. coli* O157:H7 on fresh

cut carrots after washing in chlorinated tap water at 200 ppm. Chlorine effects cell death by producing hypochlorous acid and hypochlorite ions in solution which oxidizes bacterial cell membrane components, to cause disruption of cell membrane integrity and release of cell interior (Gómez-López *et al*., 2009). Modified atmosphere packaging further restricted increase in bacterial numbers. On the other hand, restriction of utility microorganisms may have enabled survival and possibly growth of pathogens that occur in low numbers (Farber, 1991). This was evident in Salmonella survival and detection at the end of shelf life storage.

Food safety systems encompass tools that have been developed for the delivery of safe food products along the entire production, manufacturing and distribution chain, provided they are diligently applied in the design and implementation of the supply chain (Gorris, 2005). Global GAP and HACCP implemented on the farm and processing factory respectively, provide a clear guidance to hazard control and ensure due diligence (Gorris, 2005). According to the suggested coliform limit for fresh produce eaten raw (DoH, 2000), the coliform levels were unacceptably high after processing and at the end of shelf life. The South African Guidelines for environmental health officers on the interpretation of microbiological analysis of food has proposed a limit of <200 CFU/g coliform count in raw fruits and vegetables (DoH, 2000; Da Silva and Medonça, 2012). With the high coliform counts it is not surprising that Salmonella was detected in the packaged lettuce during shelf life storage.

According to the suggested coliform limit for fresh produce eaten raw (DoH, 2000), the coliform levels on the lettuce were unacceptably high. A total coliform count of up to 6.4 log CFU/g was observed for the field samples. Although the wash step reduced the ACC, IE, coliform and *S. aureus* counts with approximately 1-3 log CFU/g each, the coliform levels were still high at approximately 3-4 log CFU/g in the lettuce packs. However, although *E. coli* was present in the field lettuce samples, no *E. coli* was observed at reception in packhouse, after chilling, washing and throughout storage at 5ºC for 8 days. *S. aureus* counts in lettuce packs were < 2 log CFU/g. *Salmonella* spp. were detected on day 8 in the end of shelf life samples only. *Listeria* spp. were present in field samples, at reception and after overnight chilling, absent after washing and throughout the shelf life study.

2.2.12 NORTH WEST PROVINCE – Microbiological quality and safety of basil from a farm near Brits

Specific aim

The aim of this study was to evaluate the microbiological quality of basil in the field at harvest, after-harvest upon arrival at the packhouse and from retailers.

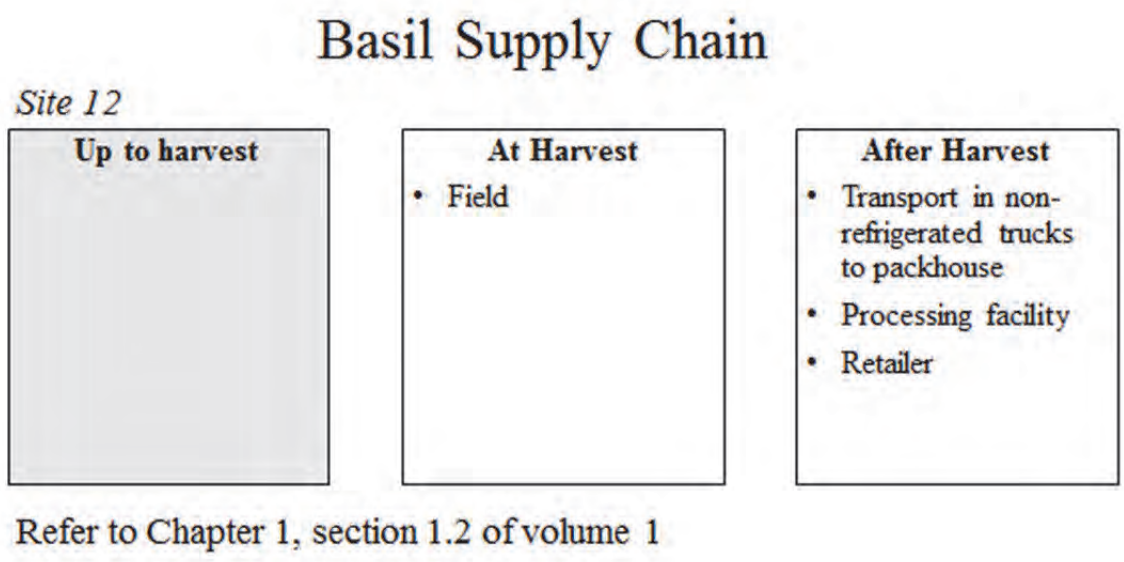
Experimental procedures

Sampling site

Basil was sampled on a commercial farm near Brits (North West Province). Irrigation water is drawn from an irrigation channel, stored in a dam and used for overhead irrigation of the basil.

Process flow

Basil samples are transported to a processing facility near Irene in Pretoria where the herbs are packed and distributed to major retailers in South Africa. Additionally, basil samples were collected from the retailers.



Basil sampling and processing

Three samples each were obtained from the field, processing facility and from a major retail outlet. The samples were aseptically collected diagonally in the basil field. One basil plant sample consisted of the leaves of three basil plants that were pooled together to add up to a total weight of 40 g. The basil packs are labelled with codes for supplier identification purposes. Basil packs with the same code were sampled at the retailers. Basil processing was as described for parsley in section 2.2.1.

Microbiological analysis

The microbiological analysis was done as described for onions in section 2.2.8.

Molecular methods

Determination of presence of *E. coli* O157:H7 and *S.* Typhimurium using multiplex PCR analysis – DNA was extracted from each of the enriched water samples using the Quick-gDNA miniprep kit (Zymo Research, California, USA). Multiplex PCR analysis was performed using pathogen specific primers and the extracted DNA as template (Cebula *et al*., 1995; Standing *et al*., 2013).

Results

Microbiological analysis

Basil

Aerobic colony counts (ACC) – The maximum ACC count was 4.5 log CFU/g for basil sampled in the field (at harvest) and the minimum count was 3.2 log CFU/g for basil sampled at the packhouse entrance after harvesting (Table 30).

Fungi – The maximum fungal count was 2.5 log CFU/g for the field basil samples and the minimum was 0.7 log CFU/g for basil sampled at the packhouse entrance (Table 30).

Yeast – The maximum yeast count was 3 log CFU/g and the minimum 2.6 log CFU/g for field basil samples (Table 30).

Coliforms and E. coli – Maximum of 4.9 log CFU/g for field basil samples and a minimum of 3 log CFU/g for basil from the retailer. The *E. coli* count for all the basil samples were zero.

E. coli O157:H7 – None detected using multiplex PCR analysis. Two of the basil samples collected at harvest tested positive for the presence of *S.* Typhimurium. Both samples were collected at the point entering the packaging line and were taken at the beginning of the day.

Table 30 Summary of microbiological analysis results obtained for basil.

Produce	Average microbial counts log CFU/g				
	Aerobic bacteria (ACC)	**Fungi**	**Yeast**	**Coliform**	***E. coli***
Field	4.55 (0.25)	2.5 (0.06)	3.01 (0.4)	4.93 (0.21)	0.00 (0)
Packhouse	3.31 (0.56)	2.23 (0)	2.55 (0.44)	3.1 (0.71)	0.00 (0)
Retailer	4.15 (0.33)	1.98 (0.1)	2.85 (0.39)	3 (0.42)	0.00 (0)

Discussion and conclusion

The microbiological quality of basil was unacceptable since coliform, yeast and mould counts for basil were higher than the DoH (2000) specifications for ready-to-eat fruit and vegetables. Furthermore, *Salmonella* Typhimurium was present in some of the packhouse basil samples and could pose a health risk, since basil is often consumed raw. The pathogen was however not detected in any of the packaged ready-to-eat basil samples from the retailers.

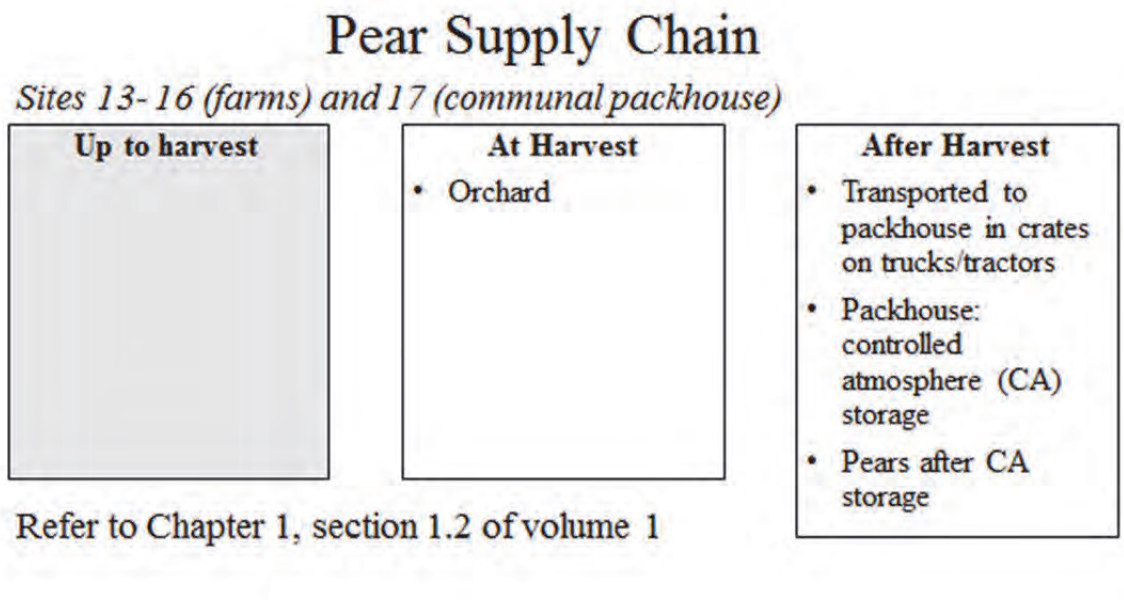
2.2.13 WESTERN CAPE PROVINCE – Microbiological quality and safety of pears from four commercial farms

Specific aim:
The aim of this study was to evaluate the microbiological quality of pears from the orchard, upon arrival at the packhouse after drenching and after modified atmosphere storage in the packhouse.

Experimental procedures
Experimental sites

- Four commercial pear production farms in the Western Cape
- Pears (*Pyrus communis* cv Packham's Triumph) sent to a communal packhouse from the four farms were sampled after drenching on arrival and again after controlled atmosphere (CA) storage.

Process flow
After harvesting in the orchards, the pears are transported in crates on trucks to a central packhouse. At the packhouse the pears are drenched once with water containing 75 ppm chlorine. After drenching the pears are stored in controlled atmosphere (CA) for 14 weeks, at oxygen levels set at 1.5% (variation 1.2-2.0%), carbon dioxide levels at 1.5% (consistency held with the aid of scrubbers) and temperature set at -0.5°C (with variation at front and back of the room 0.2°C-minus 0.7°C), where after the pears are exported.



Pear sample collection and processing
Four replicates (five pears each) were sampled from the orchards of each of the four farms, after drenching at the packhouse as well as after CA storage in the communal packhouse. The pears, picked earlier the same day, were sampled randomly from crates from each of the four

farms after drenching at the packhouse. The pear surface temperatures were data logged (Extech Instruments, IR Thermometer).

The respective pear samples were placed into 1 litre Ringer's with 0.02% Tween 80 solution, the displacement volumes determined, and sonicated in a Digital Ultrasonic Cleaner (EUmax®, Labotec, Model no. UD200SH-6L) for 5 min to remove the surface bacteria for enumeration purposes. After removal of the pears, the solution was filtered, using 0.45µm filter membranes.

Microbiological analysis

Each of the filter membranes were cut into small pieces, added to 9 mℓ peptone buffered water [PWB] (3M, Merck, South Africa), vortexed and serially diluted from 10^{-1} to 10^{-6} in 9 mℓ tryptone soy broth (TSB). Total viable bacterial, coliform/*E. coli* and fungi/yeast counts were determined as described in section 2.2.8 for onion analysis.

Selective enrichment for determining pathogen presence/absence

Each of the pear filtered samples were enriched for determining the presence of *E. coli* (including *E. coli* O157:H7) and *Salmonella* by incubation of the 9 mℓ 3M PBW with the filter membranes at 37 °C for 24 hours. Additionally, a millilitre of the 3M PBW broth was transferred into a 9 mℓ tube of 3M *Listeria* selective broth for enrichment purposes and incubated at 37°C for 24 hours.

Subsequently, a loopful of each of the pear samples (3M PBW enriched) were streaked onto eosin methylene blue differential medium (EMB) (Benton, Dickson and Company) for *E. coli* detection, *Salmonella* brilliance medium (Oxoid) agar for *Salmonella* detection and *Listeria* selective agar (Oxoid) colonies for Listeria detection.

Identification of presumptive pathogens using Matrix Assisted Laser Desorption Ionisation-Time of Flight (MALDI-TOF)

Single presumptive pathogen colonies isolated from the selective chromogenic media after selective enrichment were identified using Matrix Assisted Laser Ionisation-Time of Flight (MALDI-TOF) as described for parsley in (section 2.2.1).

Molecular analysis

Escherichia coli O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. Enterica serovar Typhimurium presence was determined using multiplex PCR analysis as described by Standing *et al*. (2013).

Molecular Detection System (MDS) analysis

The 24h 3M PBW and 3M *Listeria* specific enrichment broths were used to determine the presence/absence of *E. coli*, *Salmonella* spp. and *Listeria* spp. using the respective 3M MDS kits: 3M Molecular Detection Assay *Salmonella* (AOAC RI Certificate 031208, April 2012), 3M Molecular Detection Assay *E. coli* 0157, including H7, (AOAC RI Certificate 071202,

July 2012) and 3M Molecular Detection Assay *Listeria* (AOAC RI Certificate 081203, August 2012).

Results

Microbiological analysis of the pears

Aerobic colony counts (ACC) – The counts ranged between a minimum of 2.2 CFU/cm^2 and a maximum of 3.5 log CFU/cm^2 for the orchard, after drenching and after CA storage pear surfaces (Table 31).
Coliforms and E. coli – Coliform counts were low, i.e. a maximum count of 1.9 log CFU/ cm^2 was obtained. *E. coli* counts were zero for the orchard, after drenching and after CA storage pear surfaces (Table 31).
Yeast and fungi – After drenching the combined yeast and mould counts were lower when compared to the orchard samples, the numbers increased again after CA storage. The counts ranged between a minimum of 1.9 log CFU/ cm^2 to a maximum of 5.9 log CFU/ cm^2 (Table 31).
E. coli O157 (including H7) – none detected using 3M MDS and multiplex PCR analysis.
Salmonella spp. – none detected using 3M MDS.
Listeria spp. – detected in two pear samples only out of 36 pear samples analysed.

Table 31 Summary of microbial counts measured for pear surfaces from four commercial farms in the Western Cape

Pear farms	Sample source	Microbial counts log CFU/cm^2				
		Bacteria	**Fungi**	**Yeast**	**Coliforms**	***E. coli***
Farm 1	Orchard	2.75 (0.33)	2.19 (0)	1.29 (0)	1.65 (0.55)	0.05 (0)
	After Drench at communal packhouse	2.15 (0.53)	0.45 (0)	0.58 (0)	1.46 (1.23)	0.05 (0)
	After CA	3 (0.22)	1.24 (0)	2.6 (0)	1.7 (0.55)	0 (0)
Farm 2	Orchard	3.2 (0.5)	3.09 (0)	0.56 (0)	1.82 (0.97)	0.05 (0)
	After Drench	2.84 (0.45)	1.05 (0)	2.01 (0)	1.54 (1.13)	0.05 (0)
	After CA	2.56 (0.56)	1.04 (0)	2.32 (0)	1.25 (0.39)	0.05 (0)
Farm 3	Orchard	3.53 (0.3)	2.67 (0)	3.21 (0)	1.92 (0.89)	0.05 (0)
	After Drench	3.32 (0.4)	3.43 (0)	0.05 (0)	0.89 (0.35)	0.05 (0)
	After CA	2.81 (0.82)	1.23 (0)	3.15 (0)	0.27 (0.36)	0.05 (0)
Farm 4	Orchard	3.25 (0.41)	2.35 (0)	3.05 (0)	0.89 (0.26)	0.05 (0)
	After Drench	3.09 (0.48)	3.63 (0)	0.05 (0)	1.09 (0.4)	0.05 (0)
	After CA	2.36 (0.49)	1.39 (0)	2.67 (0)	1.1 (0.15)	0.05 (0)

CA= Controlled atmosphere storage of oxygen levels set at 1.5% (variation 1.2-2.0%), carbon dioxide levels at 1.5%

Discussion and conclusion

The *E. coli* counts in the irrigation water from the four commercial farms ranged from zero to 2.3 log CFU/100 mℓ, which is within the DWAF and WHO irrigation water guidelines for produce not to be eaten raw. *E. coli* O157 (including H7) and *Salmonella* spp. were detected in 2/57 water samples, and detected in 2/57 water samples, and *Listeria* spp.in 6/57 water samples. The pH of the irrigation water samples were within the guidelines set by DWAF's Target Water Quality Range (TWQR) of pH 6-pH 9 (DWAF, 1996).

The coliform counts for the fruit were lower when compared to most of the vegetable products and complied with the DoH guidelines. The *E. coli* count zero, and *E. coli* O157 (including H7) and no *Salmonella* spp. were detected in any of the pear fruit samples. Although *Listeria* spp. were detected in one enriched pear sample using the 3M MDS, *Listeria monocytogenes* specifically was not detected in any of the samples after streaking onto chromogenic media and identification using MALDI-TOF analysis. Recently Holfelder *et al*. (2013) reported that chromogenic media gave variable results and should be combined with other identification methods, which is in agreement with the findings of this study.

2.2.14 GAUTENG – DETECTION OF FOODBORNE PATHOGENS AND QUANTIFICATION OF MICROFLORA ON VARIOUS FRESH PRODUCE OBTAINED FROM RETAILER

Specific aim

The aim of this study was to determine the microbiological safety of fresh produce by assessing for the presence of foodborne pathogens in the retail environment. Nine different commodities were collected from four different retailers in the Pretoria, Gauteng, area in South Africa.

Experimental sites

Four well known retailers in Pretoria, Gauteng, South Africa.

Experimental procedures

Sample collection

Samples were collected from four prominent retailers in Pretoria, Gauteng province, South Africa. Oranges, lemons, strawberries, tomatoes, cucumbers, mushrooms, lettuce, spinach, rocket, potatoes and spring onions were purchased from the selected retailers. Retailer product packaging and storage condition are listed in Table 32. After purchasing, samples were immediately transported to the Plant Pathology Laboratories, University of Pretoria, where they were stored at refrigeration temperatures (4-7ºC) until processing commenced. Good laboratory practices and antiseptic techniques were applied during this study to retain product quality and prevent cross-contamination.

Sample processing

***Oranges, lemons, tomatoes and potatoes*:**

Five replicates (each replicate consisting of a single fruit or tuber) of the above mentioned commodities, were weighed and suspended into 500 mℓ quarter strength Ringer's solution amended with 0.02% Tween 80 (v/v). Samples were sonicated in an ultrasonic bath (Ultrasonic Manufacturing Company (Pty) Ltd., Johannesburg) at 25°C for 30s. The fruit were removed and the liquid solution was concentrated through a 0.45 µm nitrocellulose membrane filter using a Millipore Filtration Manifold and vacuum pump (Millipore, CC). Filter discs were aseptically cut and transferred into nine millilitres of tryptone soy broth (TSB) and serial dilutions prepared.

Strawberry, cucumber, mushroom, lettuce, spinach, rocket and spring onion:

Ten grams of fresh produce sample were weighed off and transferred into sterile stomacher bags containing 90 mℓ TSB, homogenized using a stomacher (Seward, Labotec, Johannesburg) at 240 rpm for 2 min. After maceration, the sample solution was transferred into sterile Erlenmeyer flasks, which represented the 10^{-1} dilution and a tenfold dilution series was made.

Quantification of microbial populations

The enumeration of the total viable bacterial, fungal and yeast populations were performed as described previously (section 2.3.1). The total number of colonies isolated from strawberry, cucumber, mushroom, lettuce, spinach, rocket and spring onion were recorded and expressed as the average log CFU's /gram. Colonies quantified from oranges, lemon, tomato and potato were expressed as the average log CFU's per centimetre square (cm^2) (Collignon and Korsten, 2010). The volume displaced (vd) for each fruit was converted to area (A) (square centimetres).

Detection of foodborne pathogen

Samples were enriched for *E. coli*, *Salmonella*, *Listeria* and *S. aureus* detection as described by Standing *et al*. (2013). Presumptive pathogenic colonies isolated from selective chromogenic media were purified and the identities confirmed using Matrix Assisted Laser Desorption Ionisation-Time of Flight (MALDI-TOF) (Bruker Daltonics, Johannesburg, South Africa).

Molecular analysis

DNA was extracted from the enrichment broths using Triton-X 100 DNA extraction according to the method of Wang and Slavik (2005). Multiplex PCR analysis was done as reported by Standing *et al*. (2013).

Statistical analysis

All analysis were done in triplicate. Results obtained for each repeat were analysed together, therefore 10 replicates were analysed. Statistical analysis was performed on log CFU/cm^2 and log cells/cm^2. Data was analysed using SAS 9.2 for Windows (SAS Institute Inc., Cary United

States of America). A one-way analysis of variance was used to determine the difference in pathogen titers present on fruit surfaces. Means were analysed using the least significant difference (using the Fischer test) at a 5% level of significance.

Table 32 Storage conditions and packaging of commodities from the retailers

	Retailer 1		Retailer 2		Retailer 3		Retailer 4	
Fruit	***Packaging***	***Storage conditions***	***Packaging***	***Storage conditions***	***Packaging***	***Storage conditions***	***Packaging***	***Storage conditions***
Orange	Mesh	Non-refrigerated	Pre-packed	Non-refrigerated	Pre-packed	Non-refrigerated	Pre-packed	Non-refrigerated
Lemon	hand selection, plastic bag	Non-refrigerated	Pre-packed	Non-refrigerated	Plastic bag	Non-refrigerated	Pre-packed	Non-refrigerated
Strawberry	Punnets	Non-refrigerated	Pre-packed	Refrigerated	Punnets	Refrigerated	Punnets	Refrigerated
Tomato	Plastic bag	Non-refrigerated	Pre-packed	Non-refrigerated	Plastic bag	Non-refrigerated	Plastic bag	Refrigerated
Cucumber	plastic wrap	Refrigerated	Pre-packed	Refrigerated	Plastic wrap	Refrigerated	Plastic wrap	Refrigerated
Vegetable	***Packaging***	***Storage conditions***	***Packaging***	***Storage conditions***	***Packaging***	***Storage conditions***	***Packaging***	***Storage conditions***
Mushroom	Pre-packed	Refrigerated	Pre-packed	Refrigerated	Pre-packed	Refrigerated	Pre-packed	Refrigerated
Leafy vegetable	***Packaging***	***Storage conditions***	***Packaging***	***Storage conditions***	***Packaging***	***Storage conditions***	***Packaging***	***Storage conditions***
Lettuce	Pre-packed	Refrigerated	Pre-packed	Refrigerated	Pre-packed	Refrigerated	Pre-packed	Refrigerated
Spinach	not packed	Non-refrigerated	Pre-packed	Refrigerated	Not packed	Non-refrigerated	Pre-packed	Refrigerated
Rocket	Pre-packed	Refrigerated	Pre-packed	Refrigerated	Pre-packed	Refrigerated	Pre-packed	Refrigerated
Tuber	***Packaging***	***Storage conditions***	***Packaging***	***Storage conditions***	***Packaging***	***Storage conditions***	***Packaging***	***Storage conditions***
Potato	Pre-packed, washed	Non-refrigerated	Pre-packed	Non-refrigerated	Pre-packed	Non-refrigerated	Pre-packed	Non-refrigerated
Bulb	***Packaging***	***Storage conditions***	***Packaging***	***Storage conditions***	***Packaging***	***Storage conditions***	***Packaging***	***Storage conditions***
Spring onion	Pre-packed	Refrigerated	Pre-packed	Refrigerated	Pre-packed	Refrigerated	Pre-packed	Refrigerated

Results

Total aerobic colony counts (ACC)

Bacteria, mould, and yeast were detected on all of the commodities from all of the retailers (Table 33 and Table 34). The variation of counts go from the minimum detectable level (<log 0.3) to the maximum detectable level (>log 8).

Table 33 The microbial load of fresh produce (per gram) at the time of purchase from four different retailers.

Commodity	Retailer	Quantification log (CFU/g)		
		Bacteria	**Mould**	**Yeast**
Cucumber	Retailer 1	3.48 **A**	1.07 **A**	0.64 **A**
	Retailer 2	2.58 **A**	3.01 **A**	1.62 **A**
	Retailer 3	4.18 **A**	1.65 **A**	1.28 **A**
	Retailer 4	3.84 **A**	1.28 **A**	<0.3
Lettuce	Retailer 1	5.48 **AB**	2.57 **A**	3.62 **B**
	Retailer 2	4.81 **B**	2.5 **A**	4.25 **AB**
	Retailer 3	4.76 **B**	2.22 **A**	4.20 **AB**
	Retailer 4	5.71 **A**	1.79 **A**	5.26 **A**
Spinach	Retailer 1	6.80 **A**	2.99 **AB**	4.19 **A**
	Retailer 2	5.23 **B**	2.28 **B**	4.47 **A**
	Retailer 3	6.39 **A**	3.27 **AB**	4.76 **A**
	Retailer 4	5.25 **B**	4.52 **A**	3.71 **A**
Spring Onion	Retailer 1	5.11 **A**	1.32 **B**	5.51 **AB**
	Retailer 2	5.85 **A**	2.07 **AB**	5.23 **B**
	Retailer 3	6.49 **A**	2.32 **AB**	6.58 **A**
	Retailer 4	6.04 **A**	5.41 **A**	5.75 **AB**
Strawberry	Retailer 1	2.96 **A**	0.64 **A**	2.64 **A**
	Retailer 2	1.78 **AB**	1.23 **A**	1.93 **AB**
	Retailer 3	1.04 **AB**	1.81 **A**	1.783 **AB**
	Retailer 4	0.62 **B**	2.431 **A**	1.02 **B**
Rocket	Retailer 1	4.82 **B**	3.27 **AB**	3.53 **B**
	Retailer 2	4.14 **C**	4.30 **AB**	3.42 **B**
	Retailer 3	5.14 **B**	2.91 **B**	2.34 **B**
	Retailer 4	6.30 **A**	5.21 **A**	4.99 **A**
Mushroom	Retailer 1	>8 **A**	>8 **A**	>8 **A**
	Retailer 2	>8 **A**	>8 **A**	>8 **A**
	Retailer 3	>8 **A**	>8 **A**	>8 **A**
	Retailer 4	>8 **A**	>8 **A**	>8 **A**

Means followed by the same **letter in upper case** do not differ significantly (P <0.05) between retailers where the commodities were purchased. *LSD is the Fisher's protected t-test least significant difference at the 5% level.

Table 34 The microbial load of fresh produce (per surface area) at the time of purchase from four different retailers.

Commodity	Retailer	Quantification log (CFU/cm^2)		
		Bacteria	**Mould**	**Yeast**
Tomato	Retailer 1	3.58 **AB**	0.85 **C**	1.432 **AB**
	Retailer 2	3.74 **AB**	2.39 **A**	2.53 **A**
	Retailer 3	3.10 **B**	1.03 **BC**	0.97 **B**
	Retailer 4	4.16 **A**	2.28 **AB**	2.38 **A**
Lemon	Retailer 1	3.01 **A**	1.21 **B**	1.38 **B**
	Retailer 2	3.55 **A**	2.45 **AB**	1.02 **B**
	Retailer 3	3.11 **A**	2.67 **A**	3.47 **A**
	Retailer 4	3.24 **A**	2.81 **A**	1.78 **B**
Orange	Retailer 1	2.17 **B**	0.72 **C**	0.51 **BC**
	Retailer 2	1.63 **B**	>8 **A**	<0.3 **C**
	Retailer 3	3.86 **A**	2.71 **B**	3.86 **A**
	Retailer 4	2.02 **B**	2.02 **B**	1.38 **B**
Potatoes	Retailer 1	2.15 **C**	2.51 **A**	2.90 **B**
	Retailer 2	5.9 **A**	3.45 **A**	3.88 **A**
	Retailer 3	4.18 **B**	1.79 **A**	2.22 **C**
	Retailer 4	5.56 **A**	1.51 **A**	3.91 **A**

Means followed by the **same letter in upper case** do not differ significantly (P <0.05) between retailers where the commodities were purchased. *LSD is the Fisher's protected t-test least significant difference at the 5% level.

Staphylococcus spp. were detected from all retailers (Table 35). No definite *S. aureus* was detected on the 220 samples taken from the four retailers (Table 35). Cucumber was mainly contaminated with *Staphylococcus* spp. Fourteen of the 220 samples were positive for *Staphylococcus* spp. (Table 35).

Table 35 *Staphylococcus* spp. detected on produce from all four retailers

Organism Detected	Retailer	Commodity	Score Value 1	Score Value 2
Staphylococcus spp.	Retailer 1	Cucumber	1.739	1.51
		Cucumber	1.808	1.619
		Cucumber	1.733	0
		Cucumber	1.996	0
		Mushroom	1.826	1.88
		Mushroom	1.74	2.014
	Retailer 2	ND	-	-
	Retailer 3	Cucumber	1.896	0
		Cucumber	1.748	1.595
		Cucumber	1.702	1.621
		Mushroom	1.754	1.852
	Retailer 4	Cucumber	1.777	1.837
		Cucumber	1.945	1.826

No *Salmonella* spp. or *Listeria* spp. were detected on the 220 samples purchased. Retailer 1's spring onion and tomato, Retailer 2's cucumber, lettuce and spinach, Retailer 3's cucumber and Retailer 4's potatoes all were contaminated with *Enterobacter* spp. (Table 36). *Enterobacter cloacae* was detected on spring onion and rocket from Retailer 1, on lettuce from Retailer 2 and on cucumber and tomato from Retailer 3 (Table 36). Another *Enterobacter* sp., *E. kobei* was detected on Retailer 2's spinach and on Retailer 3's oranges (Table 36). *Enterococcus faecalis* was found to be contaminating Retailer 3's cucumber, potato and spring onions and Retailer 3's spring onions (Table 36). *E. coli* was detected from all retailers (Table 36). Lettuce, spinach and mushrooms from Retailer 1, mushrooms from Retailer 2, lettuce and mushrooms from Retailer 3 and cucumber, lettuce and rocket from Retailer 4 were contaminated with *E. coli* (Table 36). *Klebsiella* sp. was detected from spinach that was purchased from Retailer 3 (Table 36). *Klebsiella pneumonia* was detected on spinach acquired from Retailer 1, on oranges, lettuce and potatoes purchased from Retailer 3 and on lemons and potatoes from Retailer 4 (Table 36).

Table 36 Enteric organisms detected from all four retailers

Organism Detected	Retailer	Commodity	Score Value 1	Score Value 2
Escherichia coli	Retailer 1	Lettuce	2.256	0
		Spinach	2.256	0
		Spinach	2.014	2.064
		Mushroom	2.334	2.298
		Mushroom	2.322	2.349
		Mushroom	2.341	2.324
	Retailer 2	Mushroom	1.948	2.265
		Mushroom	2.273	2.246
	Retailer 3	Lettuce	2.281	0
		Mushroom	2.064	2.17
	Retailer 4	Cucumber	2.351	2.001
		Cucumber	2.275	2.252
		Cucumber	2.281	0
		Cucumber	2.374	0
		Lettuce	2.38	2.26
		Lettuce	2.285	2.252
		Rocket	2.051	2.209
Enterobacter spp.	Retailer 1	Spring Onion	2.321	0
		Tomato	1.9	1.932
	Retailer 2	Cucumber	1.84	1.825
		Lettuce	1.876	1.94
		Spinach	1.972	0
	Retailer 3	Cucumber	1.912	1.712
	Retailer 4	Potato	2.16	2.214
Enterobacter cloacae	Retailer 1	Rocket	1.827	2.104
		Spring Onion	2.42	2.348
	Retailer 2	Lettuce	1.918	2.035
		Lettuce	2.122	2.393
	Retailer 3	Cucumber	2.313	2.047
		Tomato	1.983	2.0285
	Retailer 4	ND	-	-
Enterobacter kobei	Retailer 1	Mushroom	1.791	2.24
	Retailer 2	Spinach	2.108	1.847
	Retailer 3	Orange	2.082	2.146
	Retailer 4	ND	-	-
Enterococcus faecalis	Retailer 1	ND	-	-
	Retailer 2	ND	-	-
	Retailer 3	Cucumber	2.372	2.304
		Potato	1.916	2.487
		Spring Onion	2.37	2.075
		Spring Onion	2.086	2.226
		Spring Onion	2.378	0
		Spring Onion	2.456	2.485
		Spring Onion	2.173	2.298
	Retailer 4	ND	-	-
Klebsiella spp.	Retailer 1	ND	-	-
	Retailer 2	ND	-	-
	Retailer 3	Spinach	1.843	1.824
	Retailer 4		-	-
Klebsiella pneumoniae	Retailer 1	Spinach	2.3	2.396
		Spinach	2.396	0
	Retailer 2	ND	-	-
	Retailer 3	Oranges	2.062	2.189
		Lettuce	2.308	2.252
		Potato	2.041	2.218
	Retailer 4	Lemons	2.346	0
		Potato	2.243	0

Discussion and conclusion

Microorganisms are found naturally on fresh fruit and vegetables and make up part of the epiphytic flora (Francis *et al*., 1999). These microorganisms will vary depending on the climate and season and the natural titers are between 4 and 8 log per gram (Francis *et al*., 1999). The interior of plants are usually regarded as pathogen free, but microorganisms and even pathogens can be present because of the uptake of non-sterile water by the plant. The biggest source of food spoilage comes from moulds and these are normally caused by *Penicillium* spp., *Aspergillus* spp., *Sclerotinia* spp., *Botrytis* spp. and *Rhizopus* spp. The total counts in this study ranged from 0.8 to 8.8 log CFU/g. Ruiz *et al.* (1987) found that the total counts on leafy greens found at retailers to vary between a log 4 to 6 CFU/g, a range that corresponds with the current study. Ruiz *et al*. (1987) found that about 90% of the microorganisms found on commodities studied were thought to be *Pseudomonas* spp., *Enterobacter* spp. or *Erwinia* spp. (Francis *et al*., 1999).

In this study the absence and presence of foodborne pathogens was determined on all the commodities. No *Salmonella* spp. were detected on the commodities. In previous studies, *Salmonella* had been found on salads, coriander, melons, cantaloupe, tomato, and mangoes (Tauxe *et al.*, 1997) and *Escherichia coli* had been found on lettuce and tomatoes (Tauxe *et al.*, 1997).

No *Listeria* spp. (including *L. monocytogenes*) were detected on any commodities in the present study. *L. monocytogenes* can cause serious disease in humans and its ability to grow at low temperatures is of great concern. This organism is also capable of surviving at low oxygen levels. In France, the limit for *L. monocytogenes* number is 10^2 CFU/g by the sell-by date (Francis *et al.*, 1999). In Germany, no further investigation is made if the numbers of *L. monocytogenes* is less than 10^2 CFU/g but further investigation is needed if it is above this number (Francis *et al.*, 1999). In this study all the commodities were free from *L. monocytogenes.* This organism might possibly not be able to survive on the surfaces of these commodities.

The major source of *Escherichia coli* O157:H7 is thought to be the gastrointestinal tract of animals. This organism is capable of surviving in water sources and is therefore a threat to consumers when used for direct drinking or for washing raw food (Francis *et al.*, 1999). In this study *E. coli* has been detected on lettuce, spinach, mushrooms, cucumbers, and rocket and on products from all four retailers. These commodities are all vegetables that are leafy or soft and were macerated.

Salmonella spp. are mesophiles and will grow at optimum temperatures of 35-43°C. These organisms are a risk, as they are also able to grow at low oxygen levels and will therefore survive the effect of modified atmosphere packaging. *Salmonella* sp. are frequently found in excreta and sewage and if in contact, contaminates soil and crops. Food, especially of animal origin and if exposed to sewage, is the major root of *Salmonella* infection in humans. A study in Spain showed 7.5% of whole vegetables found in the field or retailer to be contaminated with *Salmonella* (Francis *et al*., 1999). Another study in Italy found 68% of lettuce and 72%

of fennel samples found at retailers to be contaminated with *Salmonella* spp. (Francis *et al*., 1999). In Malaysia *Salmonella* was isolated from 4% of leafy greens and 20% of beansprouts (Arumugaswamy *et al*., 1994; Francis *et al.*, 1999). No definite *Salmonella* colonies were detected on any of the commodities in this study. This observation might be because the *Salmonella* might not be able to colonize any of the commodities or that it was never brought into contact with these commodities.

Staphylococcus spp. were found on all four retailer samples and cucumbers mostly contained *Staphylococcus* spp. The cucumbers are packed by hand into plastic wrap and therefore a lot of handling takes place. Some species of *Staphylococcus* are natural inhabitants of skin and therefore handling will increase the risk of contamination.

In this study *E. coli* was detected on lettuce, spinach, mushrooms, cucumbers, and rocket. No *Salmonella* spp. and *Listeria* spp. were detected on any of the retail samples. *Staphylococcus* spp. were detected in cucumbers and mushrooms, however *S. aureus* was not detected in any of the samples.

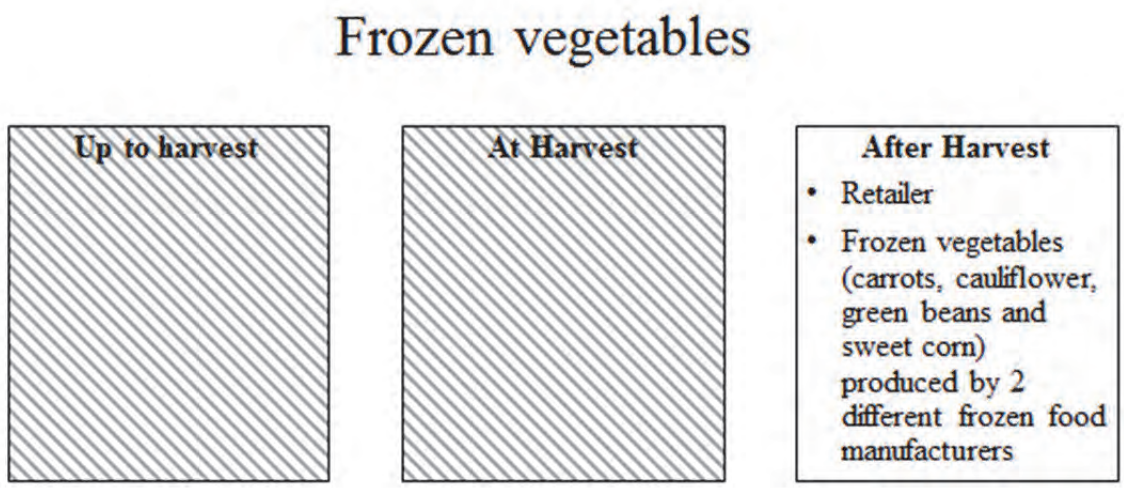
2.2.15 KWAZULU-NATAL – MICROBIOLOGICAL QUALITY OF FROZEN VEGETABLES

Specific aim

The aim of this study was to determine the microbial quality of four varieties of frozen vegetables produced by two different frozen food manufacturers in South Africa, in order to ascertain whether the quality of the vegetables is of an acceptable level. The aim was also to determine the effect of freezing on the microbiological quality of each of the vegetables over a period of time.

Experimental procedures

Sampling site



Sampling

Four varieties of packaged frozen vegetables (carrots, cauliflower, green beans and sweet corn) produced by two different frozen food manufacturers were purchased on three occasions from a well-known retailer during the study. The corners of the bags were disinfected with 70% ethanol and air-dried, prior to cutting with sterilized scissors, before the analysis.

Microbiological analysis

Enumeration of heterotrophic bacterial population (Heterotrophic plate count-HPC)

Ten grams of each sample was homogenized in 90 mℓ of 0.1% Buffered Peptone Water (BPW) in a laboratory blender for 2 min. Tenfold dilutions of the blended samples were made, spread plated onto plate count agar (PCA), incubated at 37°C for 24 hours and enumerated.

Enumeration of bacterial pathogens

Selective media were used for the isolation of specific microorganisms. HiCromform agar, *Listeria monocytogenes* Differential agar, Modified Bismuth Sulfite agar, Mannitol Salt agar and Sulfite Polymyxin Sulfadiazine agar were used for the recovery of *E. coli*/coliforms *L. monocytogenes, S.* Typhimurium, *S. aureus, Y. entercolitica* and *C. perfringens*, respectively. Colonies were enumerated after incubation of plates at 37°C for 24-48 hours. With the exception of inoculated sulfite polymyxin sulfadiazine agar plates which were incubated under anaerobic conditions, all other plates were incubated aerobically.

Identification of heterotrophic bacteria

A preliminary identification of all isolates was made, based on their cultural characteristics and morphological tests, including Gram-staining (Singleton, 2004) and spore staining (Price and Frey, 2003). Identities of the isolates were confirmed using 16S rDNA analysis.

16S rDNA gene sequence analysis

Total genomic DNA was extracted from pure cultures of each isolate using a QIAamp DNA Mini Kit (Qiagen, Germany). The total genomic DNA was used as a template for PCR amplification. The 16S rRNA genes of the bacterial isolates were amplified with the universal primers: 63f (5'–CAGGCCTAACACATGCAAGTC–3') and 1387r (5'–GGGCGG(A/T) GTGTACAA GGC–3') as described by Marchesi *et al*. (1998). PCR products were sequenced at Inqaba Biotech. The DNA sequences were subjected to BLAST searches at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) to identify the isolates.

Determination of the effect of freezing on the microbiological quality of frozen vegetables

One batch of samples that was purchased was refrozen at −20°C following the initial microbial analysis. These samples were analysed at weekly intervals for 2 months to monitor changes in microbial load.

Results

Microbial loads of different batches of frozen vegetables

Figures 13-18 show the microbial loads of three batches of frozen vegetables produced by two frozen food manufacturers (A and B) that were purchased at three different occasions during the study. Cauliflower produced by manufacturer A had the highest heterotrophic plate count of 7.13 log CFU/g in Batch 2 (Fig. 13). Cauliflower produced by manufacturer A (Batch 2) also exhibited the highest counts of *S. aureus* and coliforms, being 4.84 log CFU/g and 4.5 log CFU/g respectively (Fig. 15). The highest load of *Y. enterocolitica* (4.4 log CFU/g) was exhibited by the third batch of sweet corn produced by manufacturer B (Fig. 17). This pathogen was not detected in the second batch of green beans produced by manufacturer A. Both presumptive *L. monocytogenes* and *S.* Typhimurium were present at significantly lower levels than all other pathogens in all the samples analysed, with the latter organism not being detected in several samples. *E. coli* and *C. perfringens* were not detected in any of the samples analysed in this study.

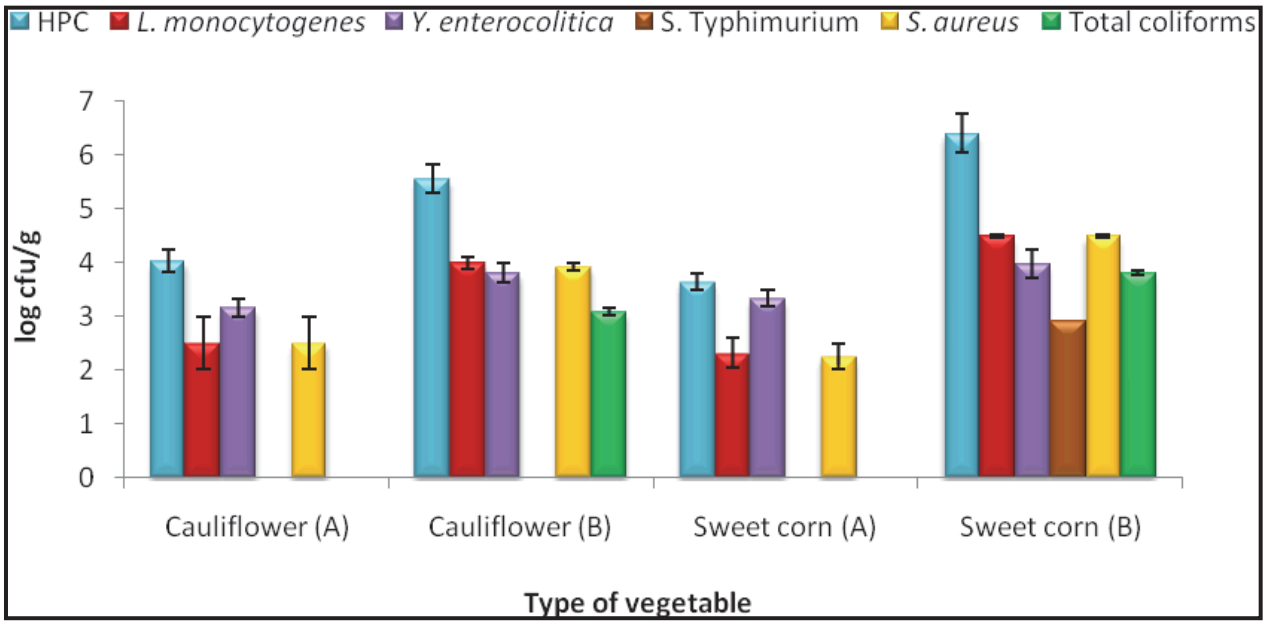


Figure 13: Microbial load of frozen cauliflower and sweet corn produced by manufacturers A and B – Batch 1.

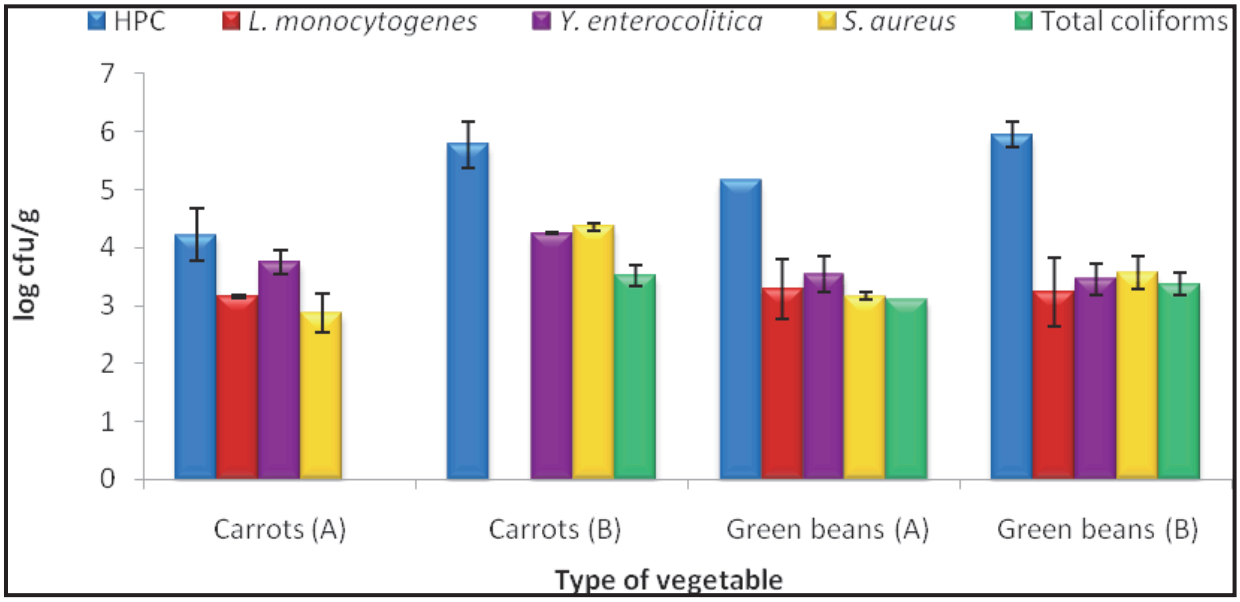


Figure 14: Microbial load of frozen carrots and green beans produced by manufacturers A and B – Batch 1.

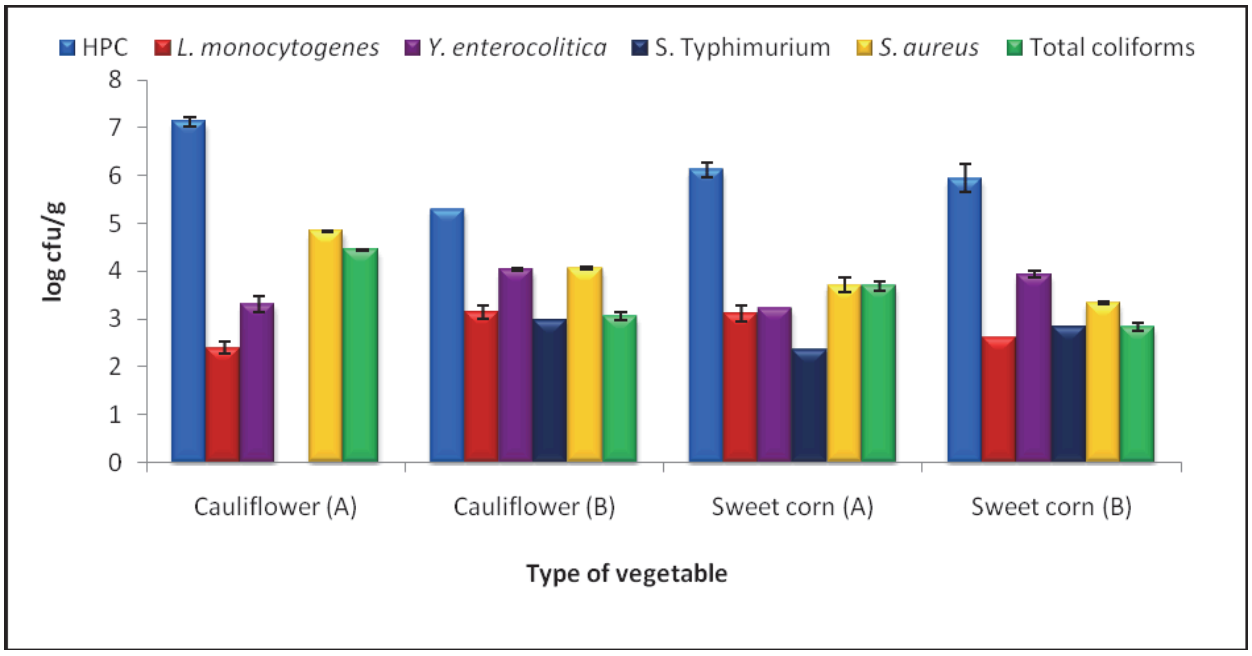


Figure 15: Microbial load of frozen cauliflower and sweet corn produced by manufacturers A and B – Batch 2.

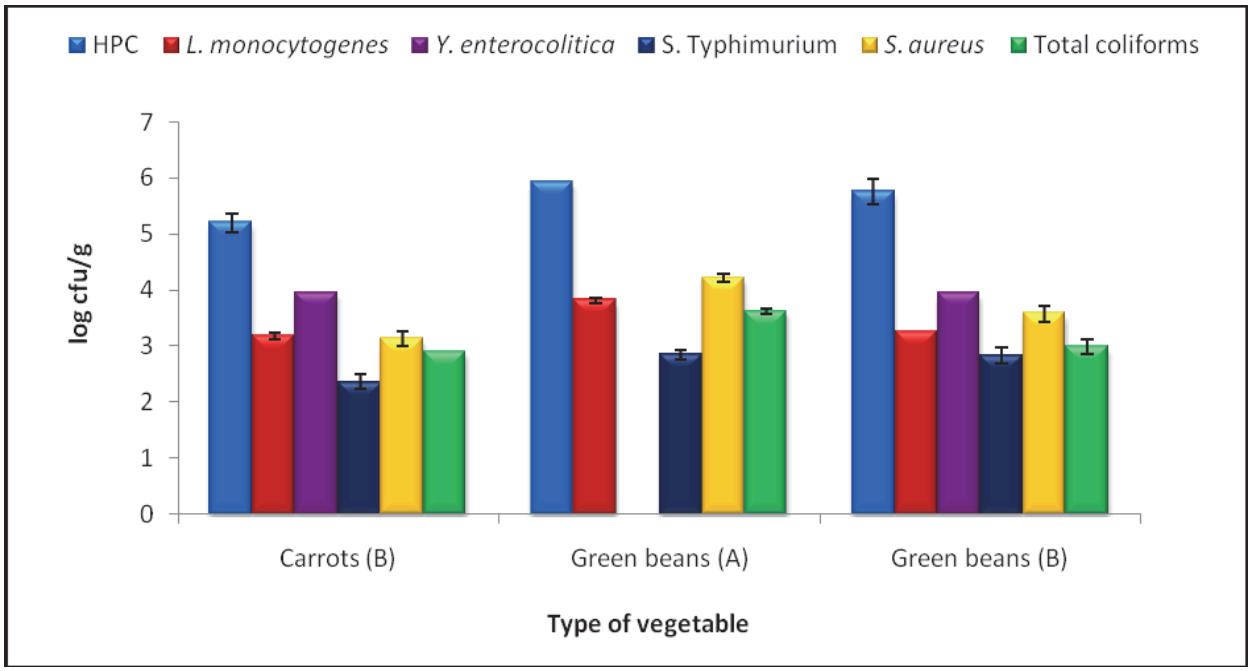


Figure 16: Microbial load of frozen carrots and green beans produced by manufacturers A and B – Batch 2.

NB: Carrots produced by manufacturer A was not in stock.

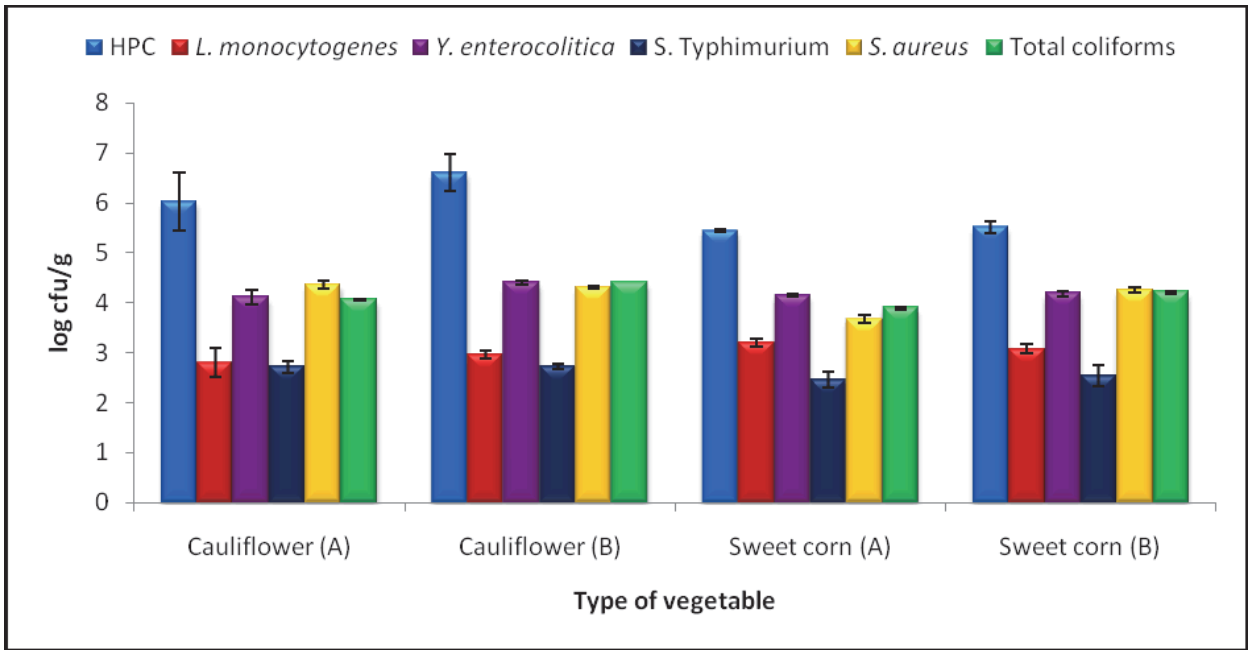


Figure 17: Microbial load of frozen cauliflower and sweet corn produced by manufacturers A and B – Batch 3.

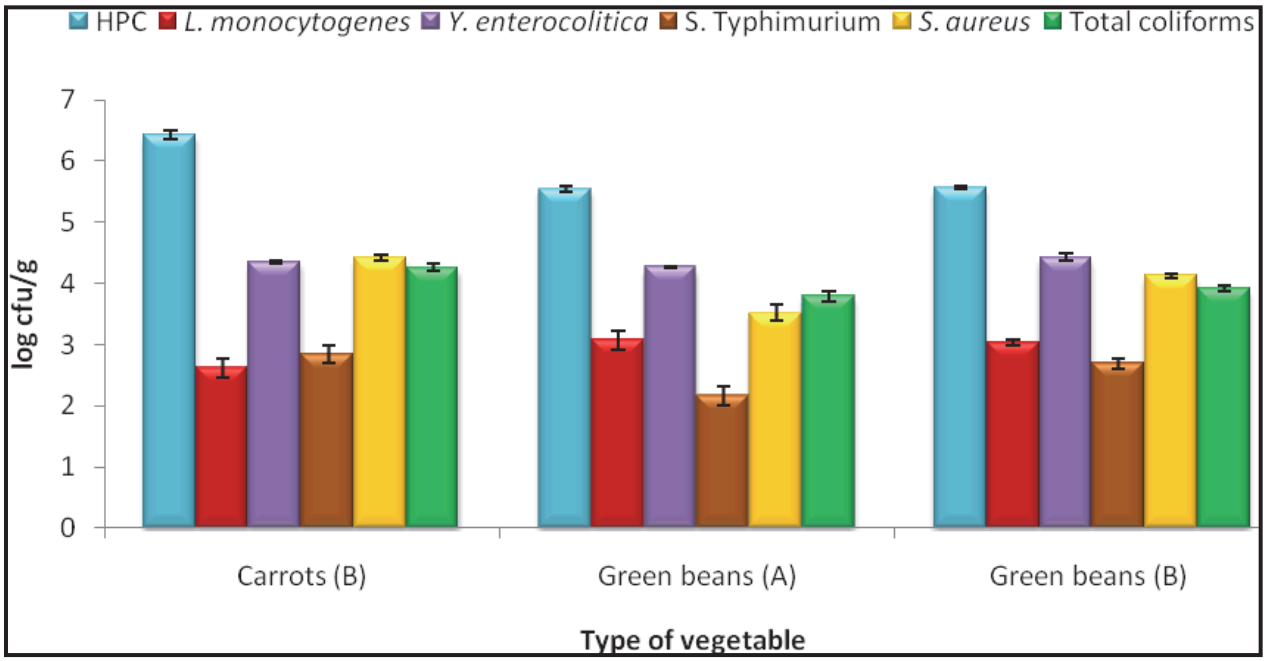
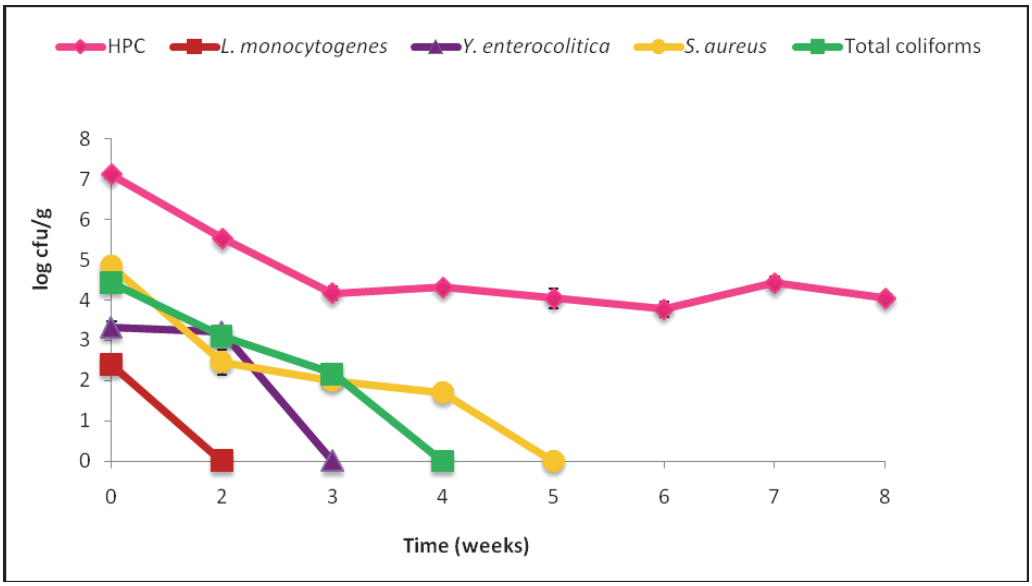


Figure 18: Microbial load of frozen carrots and green beans produced by manufacturers A and B – Batch 3.

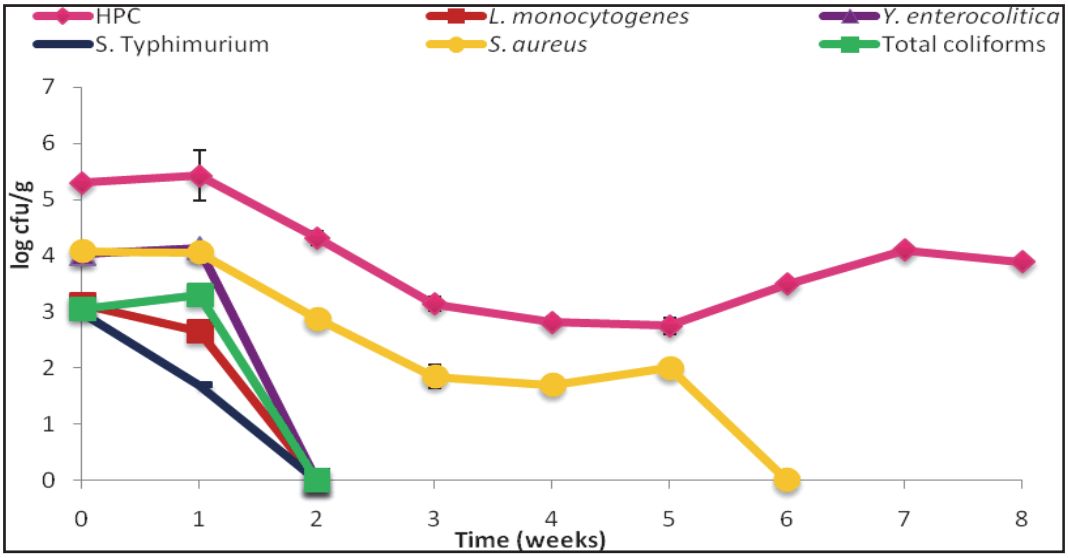
NB: Carrots produced by manufacturer A was not in stock.

The effect of freezing on the microbial load of vegetables

The effect of freezing on the microbial load of vegetables produced by manufacturers A and B are shown in Figures 19-22. As expected for vegetable products the highest initial loads were obtained for heterotrophic microorganisms in all samples analysed. These organisms exhibited the most resistance to freezing, with a significant population remaining after eight weeks of freezing. From the pathogenic species, *S. aureus* exhibited the highest initial load on all samples except for sweet corn and green beans produced by manufacturer B. *S. aureus* also exhibited the most resistance to freezing, with growth persisting for up to four to five weeks of freezing on all samples (Figs 19-22), except for green beans produced by manufacturer B, in which case a load of log 3.3 CFU/g was detected after eight weeks of freezing (Fig. 21-B). The initial loads of *L. monocytogenes* were significantly lower than that of the other pathogens and this organism exhibited the least resistance to freezing with no growth detected on samples after just one to two weeks of freezing. Initial loads of *Y. enterocolitica* were slightly higher than that of *L. monocytogenes* in all samples with similar levels of resistance exhibited by the former organism except in the case of sweet corn produced by manufacturer A where growth persisted for up to three weeks (Fig. 20-A), and green beans produced by manufacturer A (Fig. 21-A) where no growth was detected. Coliforms were detected in all samples at levels > 2.8 CFU/g and up to up to log 4.5 CFU/g *S.* Typhimurium was detected in all samples at levels < 3 log CFU/ g and up to 2.98 CFU/g except for cauliflower produced by manufacturer A where no growth was detected (Fig. 19-A). *S.* Typhimurium was shown to be susceptible to freezing with no growth detected after two to three weeks of freezing, except in the case of sweet corn produced by manufacturer A where growth was detected after six weeks of freezing (Fig. 20-A). After eight weeks of freezing, there were significant reductions in loads of heterotrophic microorganisms of 43%, 27%, 4%, 27%, 30%, 26% and 25% for cauliflower produced by manufacturer A, cauliflower produced by manufacturer B, sweet corn produced by manufacturer A, sweet corn produced by manufacturer B, green beans produced by manufacturer A, green beans produced by manufacturer B and carrots produced by manufacturer B, respectively (Figs 19-22). There was a 100% reduction in the loads of pathogenic species on all vegetables, with the exception of *S. aureus* on green beans produced by manufacturer B where a reduction of 8% was achieved following eight weeks of freezing (Fig. 21-B). In some cases, increases in microbial loads were observed, e.g. a 17% increase in the load of heterotrophic microorganisms on green beans produced by manufacturer A (Fig. 21-A) between weeks five and six and an 18% increase in the load of *S. aureus* on cauliflower produced by manufacturer B (Fig. 19-B) between weeks four and five.

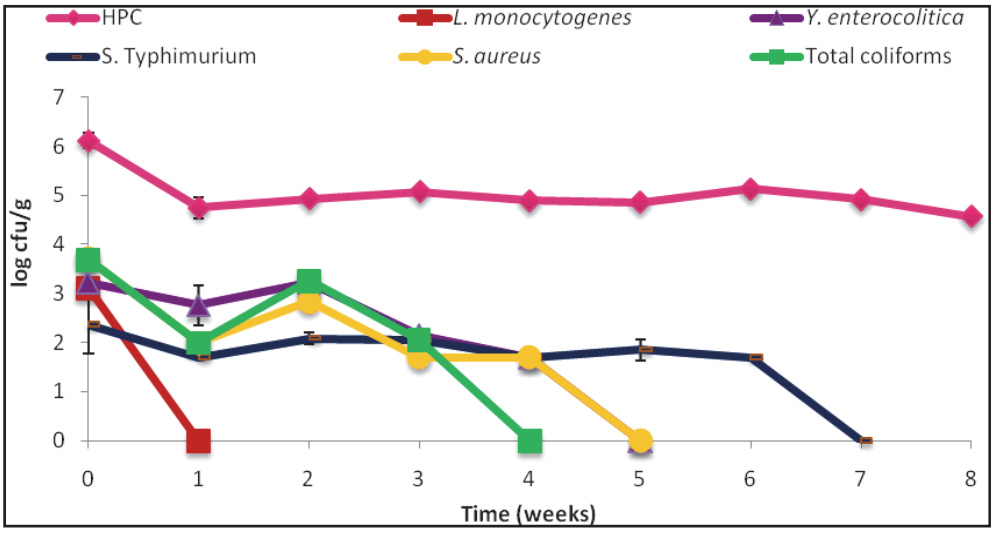


Manufacturer A

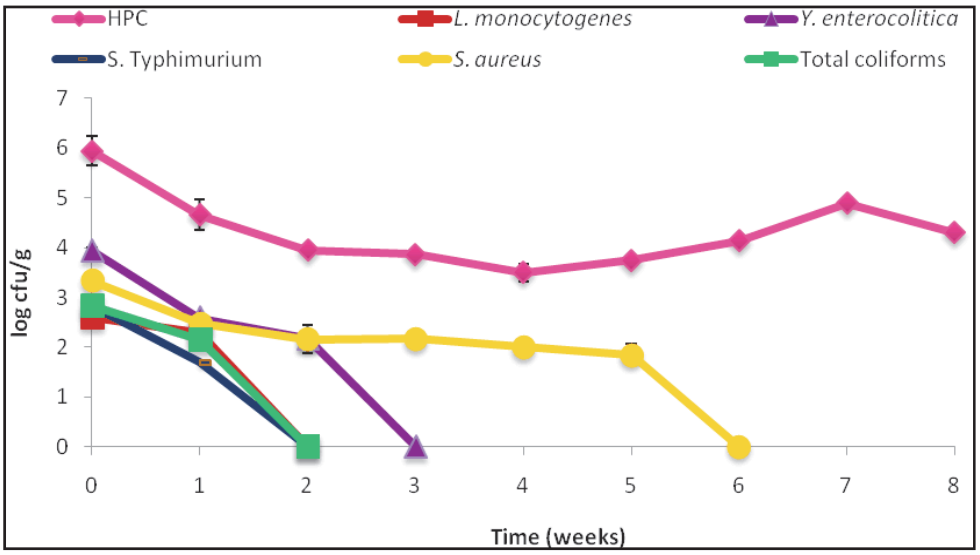


Manufacturer B

Figure 19: Microbial population changes of cauliflower produced by manufacturers A and B during frozen storage

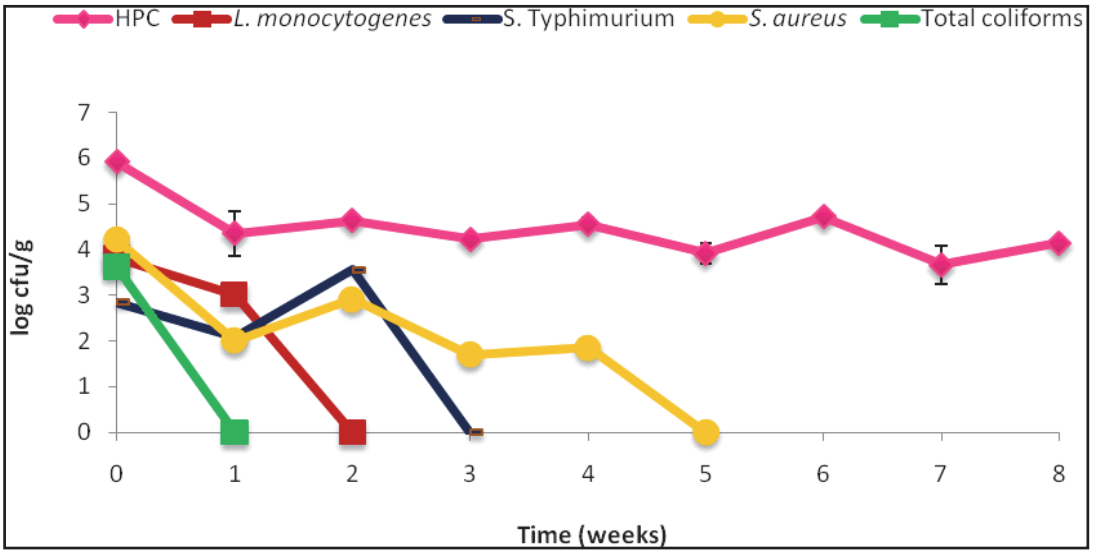


Manufacturer A

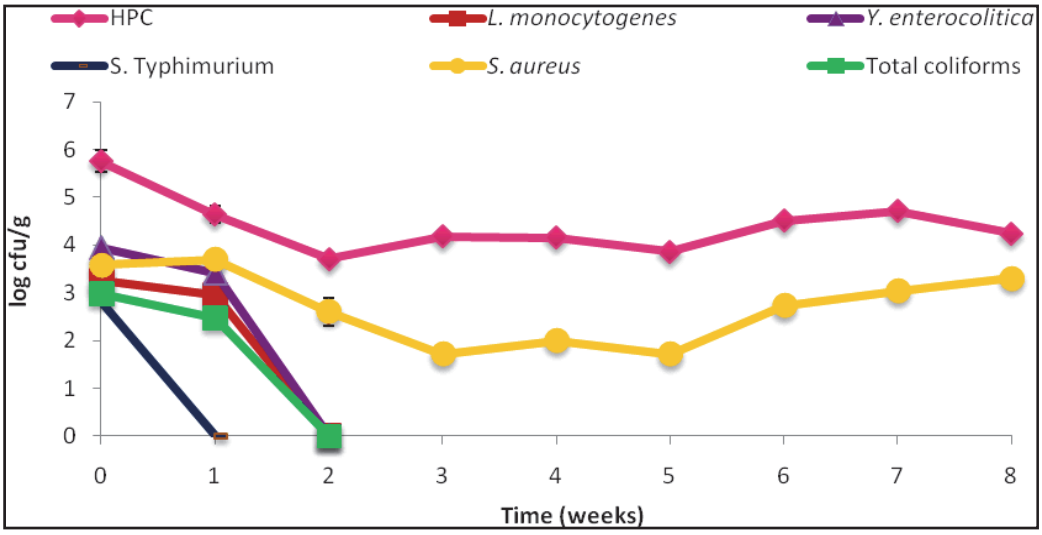


Manufacturer B

Figure 20: Microbial population changes of sweet corn produced by manufacturers A and B during frozen storage.



Manufacturer A



Manufacturer B

Figure 21: Microbial population changes of green beans produced by manufacturers A and B during frozen storage

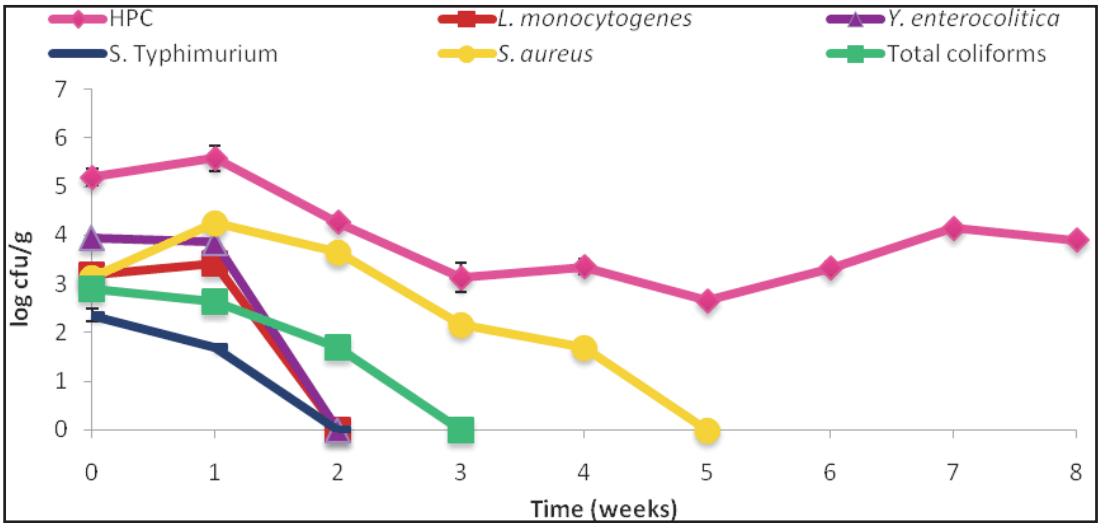


Figure 22: Microbial population changes of carrots produced by manufacturer B during frozen storage.
NB: Carrots produced by manufacturer A was not in stock.

Microorganism identification

Bacillus cereus, *Bacillus megaterium*, *Bacillus pumilis*, *Bacillus subtilis* and *Leuconostoc mesenteroides* were isolated from non-selective media.

Discussion and conclusion

Although vegetable samples were purchased locally, the two brands used for the study have high national sales figures and are sold countrywide, therefore the results can be seen as representative. The microbial loads and profiles of different batches of vegetables showed variation over the period of this study. This can be attributed to differences in the initial number of viable cells as well as the composition of the population, with some species being more resistant to freezing than others (Nussinovitch and Peleg, 2000). Other contributory factors may include variations in preparatory processes, freezing conditions and storage temperature. Therefore, the microbial loads of the individual batches are determined by both the kind of product as well as its history (Nussinovitch and Peleg, 2000).

The microbial contamination observed in the different batches of vegetable samples may have originated from a number of sources throughout production and packaging, including amongst others, contaminated irrigation or process water, application of biosolids or manure for fertilization, poor worker hygiene, and inefficient sterilization of harvesting and processing equipment (Johnston *et al*., 2005). In addition to contaminating the surface of produce, some pathogens can enter plants via wounds and stomata and can migrate through plant tissue (Buck *et al*., 2003). The internalization of *E. coli* O157:H7 from manure and irrigation water to lettuce plants, and its subsequent migration has been reported (Solomon *et al*., 2002; Wachtel *et al*., 2002). Vegetables favour the growth of bacteria owing to their high level of nutrients (Oliveira *et al*., 2008). Their high pH values (4.6 or higher) are suitable for the growth of pathogenic bacteria, particularly the Gram-negative species (Oliveira *et al*., 2008).

Vegetables like cauliflower, carrots and green beans are often sliced during processing. Slicers and cutters can be reservoirs of microbial pathogens since they may contain inaccessible sites which harbour bacteria (Garg *et al*., 1990; Francis *et al*., 1999). Cut surfaces offer a larger surface area for microbial growth and proliferation and may thus provide an ideal route for microbial infiltration (Brackett, 1994; Francis *et al*., 1999). Furthermore, moisture and exudates present on cut surfaces provide the ideal environment for rapid growth of microorganisms (Brackett, 1994). Therefore, slicing may increase the bacterial load on vegetable products. It was demonstrated that exposure of vegetables to different types of slicing methods caused an increase of six- to seven-fold in microbial populations (Brackett, 1994).

The growth of bacteria on the surface of vegetables may result in the formation of biofilms which offer protection to pathogens and reduce the effectiveness of antimicrobial agents such as chlorine which is commonly used to disinfect the surface of vegetables (Buck *et al*., 2003). Fett (2000) showed that biofilms naturally occurred on alfalfa, broccoli, cloves and sunflower sprouts and offered protection to pathogenic bacterial species such as *Salmonella* and *E. coli* O157:H7, which made their removal by antimicrobial agents difficult. In a study conducted by Lund (1983), it was shown that the inaccessibility of chlorine to microbial cells in crevices, creases, pockets and natural openings on the surface of fresh produce contributed largely to the inefficiency of chlorine in killing pathogens (Beuchat and Ryu, 1997; Lund, 1983). The fluctuating temperatures that arise due to defrosting cycles may allow for the growth of saprophytic microorganisms and this may have contributed to the microbial contamination during storage (Geiges, 1996).

Heterotrophic plate count (HPC) values for cauliflower produced by both manufacturers were significantly high in all three batches tested, with the highest value being log 7.125 CFU/g for cauliflower produced by manufacturer A (Fig. 19). These findings correlate with those obtained by Barnard *et al*. (1982) where HPC values of up to 8.7 log CFU/g were obtained for cauliflower purchased from retail markets. However, results conflict with those obtained by Schmidt-Lorenz (1982) which revealed that products consisting of larger sized pieces, including cauliflower florets of similar size to the ones tested in this study, showed negligible or no microbial growth at freezing temperatures. This discrepancy may be explained in part by the fact that the surface topography of cauliflower florets is rough and convoluted and there are numerous crevices in the lower region of the florets. These properties are conducive for the attachment of microbial cells and may hamper efforts directed towards the reduction of surface contamination (Johnston *et al*., 2005). The high microbial loads present on sweet corn, green beans and carrots (Figs 13-19) produced by both manufacturers, could be attributed to the fact that these products consisted of smaller sized pieces with a large total surface that favoured the growth and proliferation of microbial cells (Schmidt-Lorenz, 1982). With respect to the effects of freezing, it is important to note that in the retail store, the vegetables were stored at 3.4°C but when brought back to the lab, they were maintained at -20°C. This adjustment of storage temperature is of significance as studies have shown that the greatest reduction in microbial load occurs during or shortly after the sudden reduction of temperature to freezing, followed by a gradual destruction of microbial cells thereafter (Hilliard and Davis, 1918). This pattern was observed for most of the samples analysed. For

example, HPC loads for cauliflower produced by manufacturer A decreased by 22% after 3 weeks of freezing, followed by a decrease of 2.8% between week three and week eight (Fig. 19-A). However, a significant reduction in microbial load was also observed towards the latter part of the freezing period for some vegetables (e.g. a 100% reduction in the load of *S. aureus* between week five and week six for cauliflower produced by manufacturer B (Fig. 19-B).

A reduction in loads of heterotrophic microorganisms of 43%, 27%, 4%, 27%, 30%, 26% and 25% after eight weeks of freezing for cauliflower produced by manufacturer A, cauliflower produced by manufacturer B, sweet corn produced by manufacturer A, sweet corn produced by manufacturer B, green beans produced by manufacturer A, green beans produced by manufacturer B and carrots produced by manufacturer B, respectively (Figs 19-22), was observed in this study. This pattern of reduction was similar to that observed by Barnard *et al.* (1982) where it was shown that vegetables with significantly high initial heterotrophic plate counts (HPCs) (e.g. cauliflower produced by manufacturer A – Fig. 19-A) tended to have a greater percentage reduction in load after frozen storage than did vegetables with low initial HPCs (e.g. carrots produced by manufacturer B – Fig. 22).

Freezing causes a reduction in microbial load by a number of mechanisms including mechanical damage to cell walls and membranes as a result of ice crystal formation, electrolyte imbalance caused by dehydration and increased solute concentration due to formation of ice, and shrinkage of cells below a minimum critical cell volume leading to rupture of the membrane (Geiges, 1996).

In terms of the pathogenic species, it was evident that *S. aureus* was most resistant to freezing in all vegetables, with 92% of the initial population detected on green beans produced by manufacturer B after eight weeks of freezing (Fig. 21-B). These results correlate with those obtained by Georgala and Hurst (1963) who demonstrated that staphylococci were more resistant to cold storage than Gram-negative rod shaped bacteria such as *E. coli* and *salmonellae.* For some vegetables, an increase in the load of *S. aureus* was observed during the eight week freezing period (e.g. 60% increase during week five and week six for green beans produced by manufacturer B – Fig. 21-B). The resistance and increase in loads of *S. aureus* during frozen storage could be attributed to the fact that the organism is Gram-positive. Gram-positive organisms are known to be more resistant to freezing than Gram-negative species since they possess cyclopropene membrane fatty acids that contribute to membrane integrity during freezing (Smittle *et al*., 1974; Sung and Park, 1997). In view of this, the increase in microbial loads of heterotrophic microorganisms (e.g. 17% increase between weeks five and six for green beans produced by manufacturer A – Fig. 21-A) may have also resulted from the growth and proliferation of the more resistant Gram-positive organisms. Four of the five heterotrophic isolates were identified as *Bacillus* spp. which are Gram-positive, spore-forming microorganisms. Spore formers like bacilli are known to exhibit extreme resistance to freezing temperatures and may have also contributed to the observed increases in microbial loads (Lund, 2000; Archer, 2004). *B. cereus* is of particular significance in frozen foods as it is known to produce a family of cold shock proteins that enhances its survival during cold temperatures (Graumann and Marahiel, 1996). Furthermore, *B. cereus* and other *Bacillus* spp. have been associated with the outbreak of foodborne

diseases and some strains are known to proliferate and produce enterotoxins at cold temperatures (Dooley and Roberts, 2000). Therefore, their presence in food products poses a potential threat to consumer health.

The greatest attention has been focused on *L. monocytogenes* with respect to the effects of freezing (Archer, 2004). However, it was observed in this study that *L. monocytogenes* was highly susceptible to freezing since no growth was detected on all vegetables after just two to three weeks of freezing. This susceptibility could be due to the fact that higher (-18°C) freezing temperatures have been shown to be more lethal to *L. monocytogenes* than lower temperatures (-198°C) (El-Kest and Marth, 1992). Furthermore, it was also demonstrated that frozen storage of *L. monocytogenes* in foods with a pH of 4.74 (similar to pH of vegetables) at -18°C caused injury as well as a decline in the number of viable cells (Palumbo and Williams, 1991). *Y. enterocolitica, S.* Typhimurium and coliforms also exhibited susceptibility to freezing in nearly all vegetables samples with the exception of sweet corn produced by manufacturer A where growth of *Y. enterocolitica* and *S.* Typhimurium was detected after five and six weeks of freezing, respectively (Fig. 20). It has been shown that *Y. enterocolitica* is relatively sensitive to damage and inactivation by freezing processes (Stanbeck, 1996) which could account for the relatively rapid destruction of the organism that was observed for most vegetables. Although *Salmonella* spp. are known for their tolerance of freezing (Archer, 2004), resistance of the organism was only observed for sweet corn produced by manufacturer A, where growth persisted for up to six weeks of freezing (Fig. 20). The rapid decline in loads of *S.* Typhimurium that was observed for all other vegetables samples could be attributed to the low initial loads of the organism on all vegetables samples (< log 3 CFU/g for all vegetable samples). Since there were a smaller number of viable cells initially, the destruction of these cells occurred at a more rapid rate than for those organisms that were present at high initial loads (e.g. *S. aureus*). Although coliforms were present in significantly high loads for some vegetables (log 4.5 CFU/g for cauliflower produced by manufacturer A – Fig. 13, the low level of resistance observed in most cases could be attributed to the fact that that coliforms are Gram-negative which renders them more susceptible to freezing than Gram-positive organisms like *S. aureus* (Lund, 2000).

This study revealed that both heterotrophic microorganisms, coliforms and presumptive human pathogenic bacteria *(L. monocytogenes, S.* Typhimurium, *S. aureus, Y. entercolitica)* were present on frozen vegetables produced by manufacturers A and B. *E. coli* and *C. perfringens* were absent from all the vegetable samples. Freezing was shown to cause a reduction in the levels of all microorganisms (HPC, coliforms, *L. monocytogenes, S.* Typhimurium, *S. aureus, Y. entercolitica*) over eight weeks. A reduction of HPC of >25% was achieved for all samples, and in the case of the pathogenic species, growth was absent in all vegetables after eight weeks of freezing with the exception of *S. aureus* where a load of log 3.3 CFU/ g was detected on green beans produced by manufacturer B. It is evident that commercial freezing and cold storage will not render food products free of pathogenic microorganisms like staphylococci which gain access during processing which agrees with results previously reported in literature.

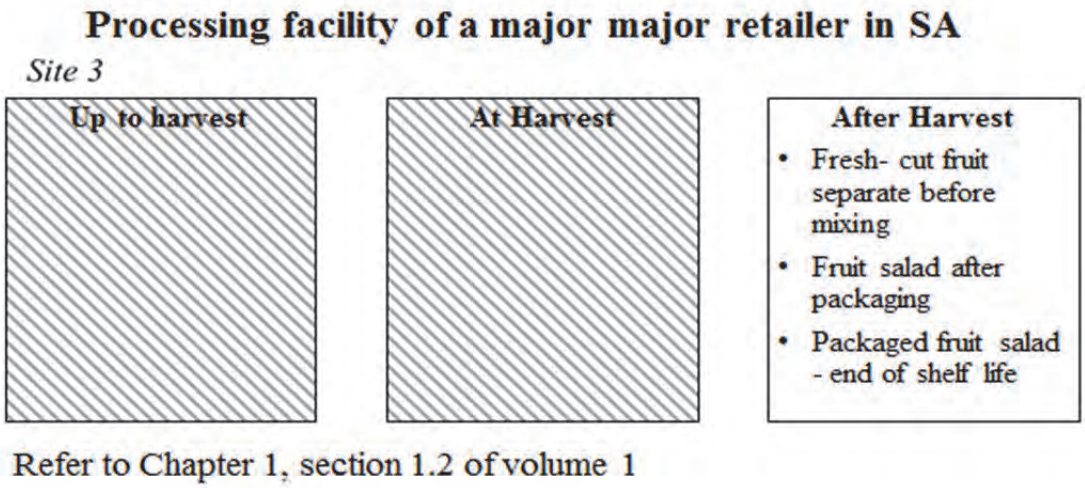
2.2.16 GAUTENG – A MICROBIOLOGICAL SURVEY OF CUT FRUIT AND PACKAGED FRUIT SALAD FROM A PROCESSING FACTORY

Specific aim

The aim of this study was to investigate the microbial quality of the various cut fruit salad components and ready-to-eat packaged fruit salad in a processing factory of South Africa, including the incidence of foodborne pathogens, i.e. *Salmonella* Typhimurium, *Listeria monocytogenes* and *Escherichia coli* O157:H7.

Experimental procedures

Sampling site



Fruit salad sampling and processing

Samples of separate cut fruit (kiwi, mango, grape, strawberry, papaya, pineapple) used in the preparation of fruit salad, as well as freshly packaged fruit salad samples were collected at the packaging facility. Three trips were made and 6 punnets were collected during each trip with the intent of testing 3 to represent the freshly packaged product. The other three were kept at typical refrigerated temperatures (5^{o}C) until "end of shelf life" to represent the quality of the fruit at "end of shelf life". For each of the samples, a 100 g of fresh cut fruit was measured out into a stomacher bag and homogenised. Twenty-five grams of the fruit pulp was added to 225 mℓ of 3M Peptone Buffered Water (PBW), 3M Petrifilm (Merck, Johannesburg, South Africa).

Microbiological analysis

Tenfold dilutions of the homogenized fruit samples were prepared and the total aerobic colony counts, coliform, *E. coli*, fungi and yeast counts determined, as described in section 2.2.1. *S. aureus* was enumerated using *Staphylococcus* count plates (3M Petrifilm).

Enrichment for detection of *E. coli* O157:H7, *Salmonella* and *Listeria* was done exactly as described for pears in section 2.2.13.

Molecular Detection System (MDS) analysis

The 24h 3M PBW and 3M *Listeria* specific enrichment broths were used to determine the presence/absence of *E. coli*, *Salmonella* spp. and *Listeria* spp. using the respective 3M MDS kits: 3M Molecular Detection Assay *Salmonella* (AOAC RI Certificate 031208, April 2012), 3M Molecular Detection Assay *E. coli* 0157, including H7, (AOAC RI Certificate 071202, July 2012) and 3M Molecular Detection Assay *Listeria* (AOAC RI Certificate 081203, August 2012).

Molecular methods

Escherichia coli O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. Enterica serovar Typhimurium presence in the enriched samples were determined using multiplex PCR analysis as described by Standing *et al*. (2013).

pH measurement of the fruit salad

The HANNA instruments HI 2210 pH meter was used to measure the pH of the homogenized fruit samples.

Results and discussion

Microbiological analysis

ACC – The counts of the end of shelf life samples were higher than counts of fresh fruit salad on day 1 (Table 37). The microbial numbers for ready-to-eat fruit salad were low when compared to the numbers reported by Althaus *et al*. (2012) for a study in Switzerland. They reported that the counts measured for 53% of the fresh cut fruits (unspecified) samples were 4-5 log CFU/g and 25% 3-4 log CFU/g. In this study the aerobic mesophilic bacterial counts measured for 29% of fresh cut fruit salad samples were < 2 log, 54% were 2-3 log CFU/g, 12.5% were 3-4 log CFU/g and only 4% were 4-5 log CFU/g. The counts measured for 40% the "end of shelf life samples" were 3-4 log CFU/g, 30% were 3-4 log CFU/g and 10% were 5-6 log CFU/g.

Fungi – ranged from zero to maximum 2 log CFU/g (Table 38).

Yeasts – present in low numbers in the fruit salad when compared to "end of shelf life", with 62% of the fresh samples showing <1 log/g and 29% 1-2 log/g. End of shelf life showed a significant increase in yeast growth with 30% having 3-4 log/g and 20% 4-5 log/g (Table 39).

S. aureus – present at very low numbers in some of the samples, i.e. < 1 log on papaya alone, 70% of fresh fruit salad samples and 60% of the end-of-shelf life fruit salad samples (Table 40).

Coliform counts – counts of 54% were 1-2 log CFU/g which is acceptable according to the DoH specifications, 33% were 2-3 log CFU/g, 12. 5% 3-4 log CFU/g (Table 41).
However the coliform counts for 70% of the end of shelf life samples had increased to > 4 log CFU/g which is unacceptably high.

E. coli – not present in any of the samples.

Table 37: Aerobic mesophilic bacterial counts of the individual components and fruit salad samples (fresh and at end of shelf life).

Aerobic bacteria Log CFU/g	Ready-to-eat Fruit salad Samples		Fresh cut fruit components					
	Fresh (n=24)	End of shelf life (n=10)	Kiwi (n=2)	Mango (n=2)	Grape (n=2)	Straw-berry (n=2)	Papaya (n=2)	Pine-apple (n=2)
<2	7	1	1	1	2	0	1	1
2-3	13	1	0	0	0	2	0	1
3-4	3	4	0	1	0	0	0	0
4-5	1	3	1	0	0	0	0	0
5-6	0	1	0	0	0	0	1	0

Table 38: Fungal counts for fresh and end of shelf life fruit salad as well as the individual components

Fungi Log CFU/g	Ready-to-eat Fruit salad		Fresh cut fruit components					
	Fresh (n=24)	End of shelf life (n=10)	Kiwi (n=2)	Mango (n=2)	Grape (n=2)	Straw-berry (n=2)	Papaya (n=2)	Pine-apple (n=2)
<1	10	5	2	2	2	0	2	0
1-2	14	5	0	0	0	2	0	0
2-3	0	0	0	0	0	0	0	2

Table 39: Yeast counts of the fresh and end of shelf life fruit salad samples, as well as each of the components individually

Yeast Log CFU/g	Ready-to-eat Fruit salad Samples		Fresh cut fruit components					
	Fresh (n=24)	End of shelf life (n=10)	Kiwi (n=2)	Mango (n=2)	Grape (n=2)	Strawberry (n=2)	Papaya (n=2)	Pineapple (n=2)
<1	15	2	2	2	1	2	2	0
1-2	7	1	0	0	1	0	0	2
2-3	2	2	0	0	0	0	0	0
3-4	0	3	0	0	0	0	0	0
4-5	0	2	0	0	0	0	0	0

Staphylococcus spp. usually reside on human skin and nasal cavities, these are often seen as a source of food contamination. Therefore little or no *Staphylococcus* spp. contamination is expected, if proper food processing safety precautions have been taken.

Table 40: *Staphylococcus* counts on individual fruit salad components, fresh and end of shelf life fruit salad samples

Log CFU/g	Ready-to-eat Fruit salad Samples		Fresh cut fruit components					
	Fresh (n=24)	End of shelf life (n=10)	Kiwi (n=2)	Mango (n=2)	Grape (n=2)	Strawberry (n=2)	Papaya (n=2)	Pineapple (n=2)
0	7	4	2	2	2	2	0	2
0-1	17	6	0	0	0	0	2	0

Results obtained with the 3M coliform/ *E. coli* count plates are summarised in Table 40. *E. coli* was found not to be present in any of the samples tested. The coliform counts of the fresh fruit salad relative to the end of shelf life fruit salad is much lower at a level of 45% at 1-2 log CFU/g. Whereas the end of shelf life 70% where >4 log CFU/g, this is a significant increase in coliforms and is also to be expected. When assessing the individual component for coliform contribution it was found that once again strawberry and pineapple had the highest levels at 2-3 log CFU/g. Mango and grape also showed potential with 1-2 log CFU/g coliforms.

Table 41: Coliform counts as determined with Petrifilm for both fresh and end of shelf life ready-to-eat fruit salad, and the individual components

Log CFU/g	Ready-to-eat Fruit salad Samples		Fresh cut fruit components					
	Fresh (n=24)	End of shelf life (n=10)	Kiwi (n=2)	Mango (n=2)	Grape (n=2)	Strawberry (n=2)	Papaya (n=2)	Pineapple (n=2)
<1	1	0	2	0	1	0	2	0
1-2	11	0	0	2	1	0	0	1
2-3	8	3	0	0	0	2	0	1
3-4	3	0	0	0	0	0	0	0
>4	1	7	0	0	0	0	0	0

pH measured for the fresh-cut fruit salad samples

The pH was measured for both the fresh and end of shelf life fruit salad samples and ranged between pH 3.5 to 3.88 (Fig. 23).

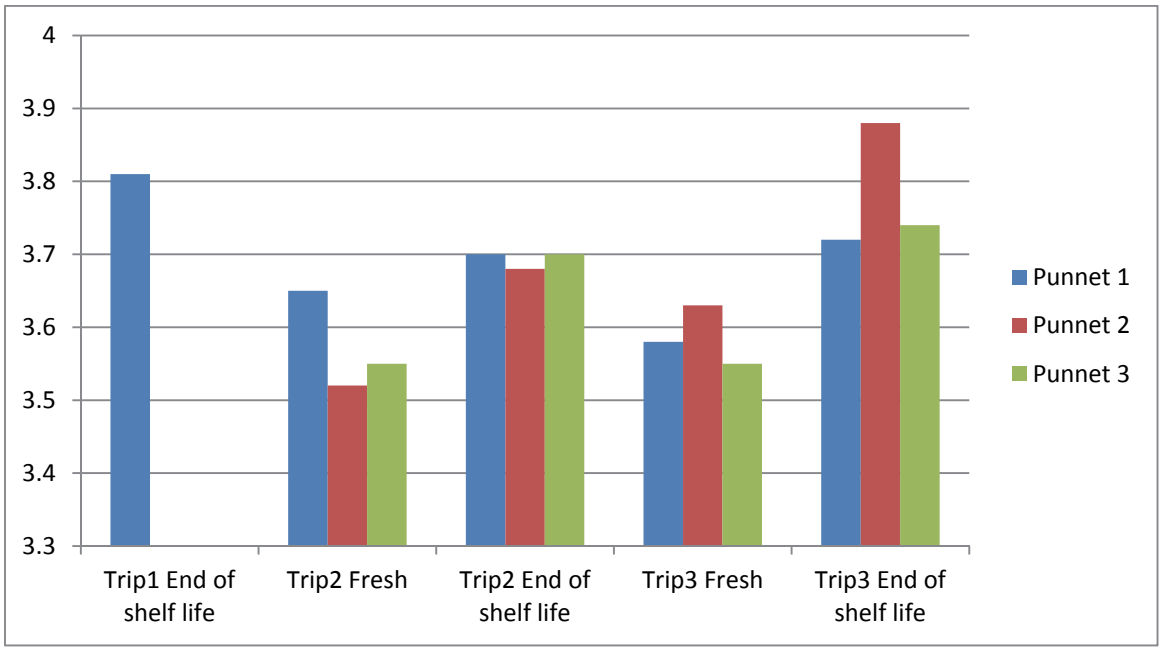


Figure 23: Graph indicating the pH values of fresh and end of shelf life fruit salad samples

3M MDS analysis to determine the presence/absence of *E. coli* O157, *Listeria* spp. and *Salmonella* spp.

No *E. coli* O157 (including H7), *Salmonella* spp. or *Listeria* spp. were detected in the samples using 3M MDS analysis.
None of the samples tested positive for either *Salmonella* Typhimurium, *Listeria monocytogenes*, *E. coli* O157:H7 using multiplex PCR analysis.

Conclusions

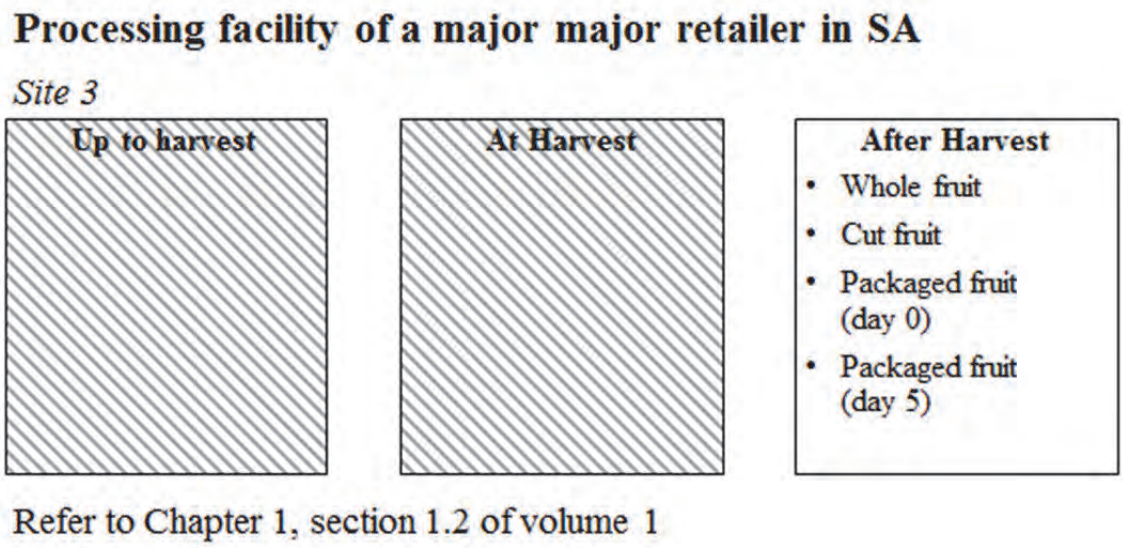
The coliform yeast and mould counts of the fresh fruit salad samples were mostly acceptable according to the DoH guidelines for ready-to-eat fresh fruit and vegetables. Although the coliform counts of 70% end of shelf life samples had increased to > 4 log CFU/g, *E. coli* was not isolated from any of the samples collected from the processing factory. *E. coli* O157 (including H7), *Salmonella* spp. or *Listeria* spp. were not detected in any of the fresh fruit salad samples. The pH was measured for both the fresh and end of shelf life fruit salad samples, ranged between pH 3.5 and 3.88.

2.2.17 GAUTENG – PREVALANCE OF MICROBIAL HAZARDS ON MINIMALY PROCESSED MANGOS AND MELONS

Specific aim
This study aimed to determine the microbial quality of minimally processed sweet melons and mangos during minimal processing including whole fruit, cut fruit, packaged fruit and packaged fruit at the end of shelf life.

Experimental procedures

Experimental site



Sampling

Sampling of mangos and melons took place on a weekly basis at a fresh produce processing plant in South Africa. During each sampling session, samples were taken in duplicates at certain stages of the minimal processing procedure, including the unprocessed fruit, the cut fruit and the packaged fruit (Fig. 24). Two packaged, minimally processed products were also analysed at the end of shelf life. These were stored at 0°C for 5 days at the Department of Food Science at the University of Pretoria before analysis.

Figure 24: The sampling stages during minimal processing

Six samples of packaged minimally processed mango (day 5) and minimally processed melon (day 5) stored at 5°C that were purchased at a local store supplied by the same processing plant and analysed immediately after purchase.

Determination of physicochemical parameters

Both the temperature and the pH of the samples were measured at the time when they were analysed in the laboratory. The temperature of the samples was measured with a portable device (Checktemp 1, Hanna Instrument, Inc. Woonsocket, R1 USA). The pH of the samples was measured using the 211 Microprocessor pH meter (Hanna Instruments, Inc. Woonsocket, R1, USA).

Microbiological analysis

Aerobic plate count

Method ISO 4833 (International Organization for Standarization, 1991) was used for the enumeration of the aerobic colonies.

Enterobacteriaceae

Enterobacteriaceae were enumerated on 3M Petrifilm and on VRBG Agar plates (Oxoid, Basingstoke, Hampshire, England).

Yeast and moulds

Yeast and moulds were enumerated on 3M Petrifilm (3M, Maplewood, Minnesota, USA)

E. coli

E.coli was enumerated according to the method ISO 16649-2 (International Organization for Standarization, 2001).

***E. coli* O157**

E. coli O157 was isolated according to the method ISO 16654 (International Organization for Standarization). Confirmation of the presumptive *E. coli* 0157 isolates was done by MALDI TOF analysis.

Presumptive *E. coli* 0157 isolates were also tested for other pathogenic *E.coli* strains with serum agglutination tests.

***Salmonella* spp.**

Salmonella spp. was isolated according to the method ISO 6579 (International Organization for Standarization, 1993). Confirmation of the presumptive *Salmonella* spp. was done at ARC Onderstepoort, Pretoria, South Africa.

Listeria monocytogenes

Listeria monocytogenes was isolated according to the method ISO 11290-1 (SABS ISO 11290-1 , 1996) Confirmation of the presumptive *Listeria monocytogenes* isolates was done by MALDI-TOF analysis.

Staphylococcus aureus

Staphylococcus aureus was isolated on 3M Petrifilm (3M, Maplewood, Minnesota, USA) and confirmed with 3M Petrifilm Staph Express Discs 6493 (3M, Maplewood, Minnesota, USA).

3M Molecular detection system

The 3M Molecular System operates by the combination of DNA amplification and bioluminescence detection technology to test if *Salmonella* spp., *Listeria* spp. or *E. coli* O157 are present in food. The system uses multiple, specific primers that target distinct regions of the pathogenic genome. If a particular pathogen is present, its genome will be amplified and real-time detection will show its presence (3M, Maplewood, Minnesota, USA). DNA was extracted from the Buffered Peptone Water (3M, Maplewood, Minnesota, USA) of all samples for determination of the presence of *Salmonella*, *Listeria* as well as *E. coli* 0157.

Fresh produce analyses

Whole fruit

A whole mango was placed in a stomacher bag containing 225 mℓ of 3M Buffered Peptone Water. After shaking the bag, the mango was removed and the Buffered Peptone Water was analysed. The melons could not be placed in a stomacher bag, since they are too big. Surface analysis was done by swabbing the entire surface with a sterile swab. This swab was placed in Buffered Peptone Water and analysed as described above for the 3M molecular detection system.

Cut fruit

Analyses of the cut fruit were done by placing 25 g of the cut fruit in 225 mℓ of 3M Buffered Peptone Water. The bag was then stomached and the filtrate was used for further analyses as described above.

Statistical analysis

Statistica Software for Windows Version 7 (Tulsa, Oklohama, USA, 2003) was used to perform a single factor Analysis of variance (ANOVA) to determine if there was a significant increase in the aerobic plate count, Enterobacteriaceae and pH respectively during processing. For this analysis, a 95% confidence interval was used and the significant level of the ANOVA test was set at $P<0.05$.

The LS means and standard deviations were also calculated for the Aerobic Plate Count, Enterobacteriaceae and pH during each processing step. The LS means and standard deviations of the Aerobic Plate Count and Enterobacteriaceae was also calculated for the 6 mango and 6 melon samples that were analysed at the end of shelf life. *Staphylococcus aureus* isolates were calculated as a percentage of the total of all the samples that were analysed.

Results

Prevalence of microbial hazards on mangos and melons during minimal processing

The aerobic colony counts obtained from the mango samples at all stages of processing ranged from 2.48 to 4.73 log CFU/g (Fig. 25). The average aerobic colony count of the full, cut, packaged (day 0) and packaged (day 5) mango samples were 3.36 log CFU/g, 3.42 log CFU/g, 3.80 log CFU/g and 4.46 log CFU/g respectivly. The aerobic colony count increased significantly during minimal processing ($P<0.05$). Levels of Enterobacteriaceae ranged from 2.45 log CFU/g to 4.81 log CFU/g. The average Enterobacteriaceae count ranged from 2.45 log CFU/g to 4.89 log CFU/g. The average Enterobacteriaceae counts of the full, cut, packaged (day 0) and packaged (day 5) mango samples were 3.29 log CFU/g, 3.60 log CFU/g, 3.64 log CFU/g and 4.27 log CFU/g respectively (Fig. 25). The Enterobacteriaceae count did not increase significantly during minimal processing ($P>0.05$). No *E. coli, Listeria* or *Salmonella* was detected. 70% of the samples analysed tested presumptively positive for *Salmonella.* These presumptive cultures were however confirmed to be *Bacillus* spp., *Klebsiella pneumoniae, Enterobacter adecarboxylata* and *Pseudomonas aeruginosa.* Twenty

per cent of the mango samples tested positive for *Staphylococcus aureus.* The average pH of the cut, full, packaged (day 0) and packaged (day 5) mango samples were 3.08, 3.43 and 4.30. Even though there was an increase, it was not significant (P>0.05) (Fig. 25).

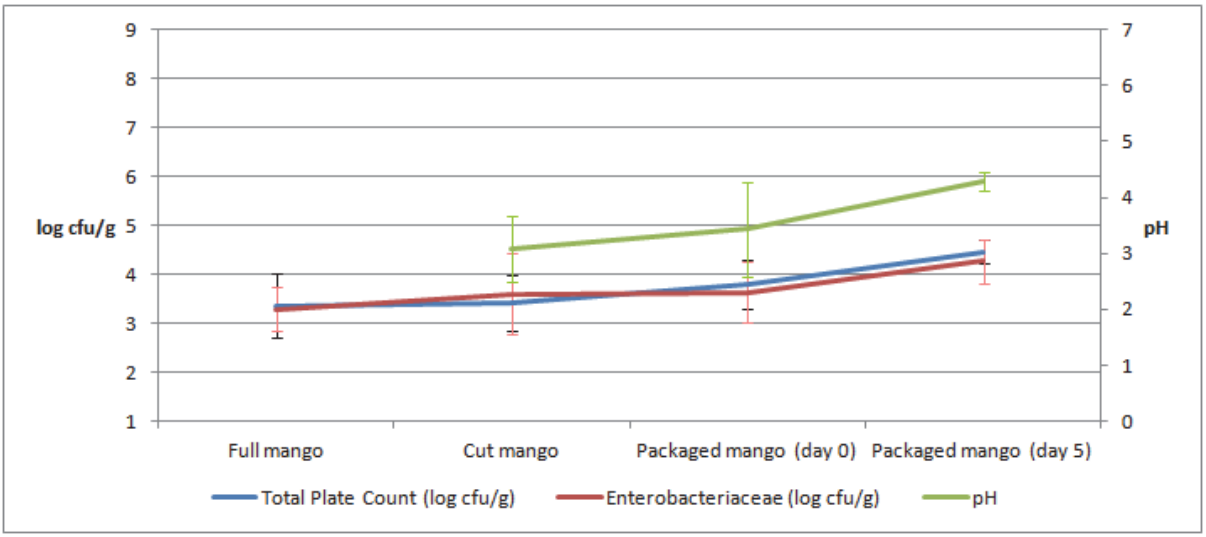


Figure 25: Total Plate Count, Enterobacteriaceae and pH during mango processing (n=32)

The aerobic colony counts obtained from the melon samples during processing and storage ranged from 3.72 to 7.51 log CFU/g (Fig. 26). The average aerobic colony count of the full, cut, packaged (day 0) and packaged (day 5) melon samples were 3.85 log CFU/g, 4.68 log CFU/g, 5.72 log CFU/g and 6.57 log CFU/g, respectively. The aerobic colony count increased significantly during minimal processing (P<0.05).

Levels of Enterobacteriaceae during processing and storage ranged from 2.40 log CFU/g to 7.10 log CFU/g (Fig. 26). The average Enterobacteriaceae count ranged from 2.40 log CFU/g to 7.10 log CFU/g. The average Enterobacteriaceae count of the full, cut, packaged (day 0) and packaged (day 5) melon samples were 3.30 log CFU/g, 4.07 log CFU/g, 4.31 log CFU/g and 5.48 log CFU/g, respectively. The Enterobacteriaceae count did not increase significantly during minimal processing and storage (P>0.05).

No *E. coli*, *Listeria* or *Salmonella* were detected in any of the samples. However, 30% of the samples analysed tested presumptively positive for *Salmonella.* These presumptive cultures however were confirmed to be *Bacillus* spp., *Citrobacter freundii*, *Klebsiella pneumoniae*, *Enterobacter asburiae*, *Enterobacter cloacae* and *Pseudomonas aeruginosa.* Seventy-five percent of the melon samples analysed were positive for *Staphylococcus aureus.* The average pH of the cut, packaged (day 0) and packaged (day 5) melons was 5.94, 5.98 and 6.50. Even though there was an inrease during processing and storage, it was not significant (P>0.05) (Fig. 26).

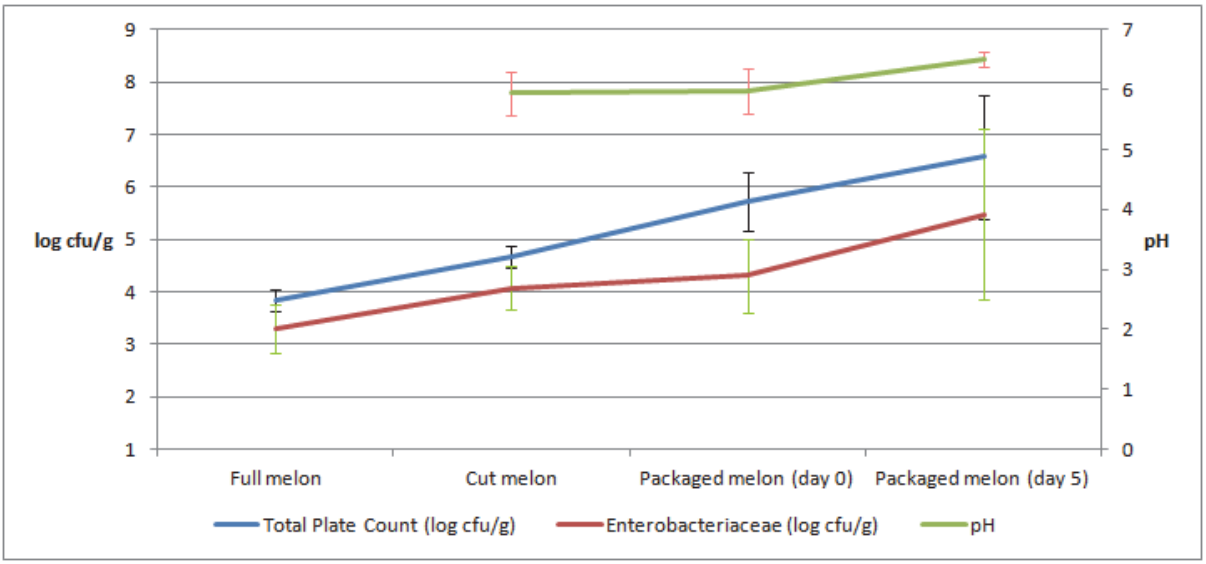


Figure 26: Total Plate Count, Enterobacteriaceae and pH during melon processing (n=32)

Prevalence of microbial hazards on fresh-cut mangos and melons at the end of shelf life

The average aerobic colony and Enterobacteriaceae counts of the 6 packaged (day 5) mango samples analysed were 5.27 log CFU/g and 4.70 log CFU/g, respectively (Table 42). The average pH for these samples was 4.03. No *E. coli*, *Listeria* or *Salmonella* was detected. 20% of these samples tested positive for *Staphylococcus aureus*. The average aerobic colony and Enterobacteriaceae counts of the 6 packaged (day 5) melon samples analysed were 5.46 log CFU/g and 5.08 log CFU/g, respectively. The average pH for these samples was 6.43 (Table 42). Presumptive *E. coli*, *Listeria* or *Salmonella* were detected but was confirmed to be *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Sixteen of these samples tested positive for *Staphylococcus aureus*.

Table 42: Comparison of means of Total Plate Count, Enterobacteriaceae and pH of the packaged mango and melon stored at 0°C and 5°C respectively

	Total Plate count		**Enterobacteriaceae**		**pH**	
Packaged mango (day5)*	4.46	±0.25	4.27	±0.45	4.30	±0.17
Packaged mango (day5)**	5.27	±0.51	4.70	±0.53	4.03	±0.27
Packaged melon (day 5)*	6.57	±1.19	5.48	±1.64	6.50	±1.22
Packaged melon (day 5)**	5.46	±0.54	5.08	±0.78	6.43	±0.27

* Packaged Mango analysed at end of shelf life that was stored at the University of Pretoria at 0°C

**Packaged Mango analysed at end of shelf life that was stored at the outlet store at 5°C

Discussion and conclusion

The significant increase in aerobic plate counts in both the mangos and melons during minimal processing can be attributed to the time that passed between the processing steps that gave the bacteria time to multiply, certain operations of the minimal processing procedure and the physical and physiochemical changes of the fruit that occur during minimal processing. The slicing operation of minimal processing destroys protective membranes and barriers of living plants, which allows the entry and contamination with microbial pathogens (Siddiqui *et al.,* 2011). Random swabs that were taken from the hands of the minimally processing operating personnel during mango and melon sampling had high aerobic colony counts ranging between 10^4-10^6 CFU/g. Therefore, microorganisms may have been transferred from the hands of the operating personnel to the fruit during the processing procedure.

Fruits consist of tissues that continue to be metabolically active even after harvest, during processing and storage (Siddiqui *et al.,* 2011). Microbial growth is greater on minimally processed products than on the intact fruit due to damaged tissues and broken cells (Ayala-Zavala *et al.,* 2010a; Ayala-Zavala *et al.,* 2010b). Cell disruption leads to the release and intermixing of enzymes and substrates that can be used by native or exogenous microorganisms to grow on the product (Siddiqui *et al.,* 2011).

In the mangos samples, the pH ranged between 3.08 and 4.30 which is characteristic for the 'Kent' cultivar (Dea *et al.,* 2010) and increased during processing which corresponds to findings in literature. During mango ripening, starch is broken down into more sugars and this lowers the percentage of acidity of the fruits (Tandon *et al.,* 1986). The decrease in acid content is due to the conversion of malic and citric acid into sugars by biochemical and physiological changes that take place within the fruit (Shafique *et al.,* 2006). This leads to a great rise in the sugar to acid ratio. The increase in sugar content may have contributed to the aerobic colony count increase as it can be used by microorganisms as a food source and these components are essential for growth and cannot be synthesized by the microorganisms themselves (Adams and Moss, 2008).

In the melon samples, the average pH was one unit greater than that of the mangos and ranged between 5.94 and 6.50 which has also been shown by Parveen *et al.* (2012) for 'Muskmelon' cultivar.

pH also increased during processing which differs from literature. According to Parveen *et al.* (2012) and others, sweet melon pH decreases during storage due to the degradation of sugars into high acid compounds (Parveen *et al.,* 2012).

The Enterobacteriaceae count increased for both the mangos and melons during processing but was not significant in the mangos. Bacteria generally grow the fastest in a pH range between 6.0 and 8.0. Bacterial growth is possible at lower pH, but it occurs at a slower rate. Yeasts grow the fastest in a pH range of 4.5 and 6 and filamentous fungi grow the best at a pH between 3.5 and 4 (Adams and Moss, 2008). The increase in microbial population during minimal processing on the mangos was therefore probably dominated by yeasts and

filamentous fungi capable of growth and survival in lower pH ranges (Adams and Moss, 2008). The yeast and moulds could not be enumerated due to an error in methodology which resulted in faulty counts. In contrast to the mangos, the increase in microbial population on the melons was probably dominated by bacteria, which thrive at higher pH values (Adam and Moss, 2008).

The significant difference in both aerobic plate count and Enterobacteriaceae count between the packaged melon and mango samples stored at 0°C and those stored at 5°C is probably due to the difference in storage temperature. Lower temperatures are known to slow down or completely inhibit growth. 5°C is the minimum temperature at which the majority of mesophilic microbes are still able to proliferate but below this temperature, growth is generally inhibited (Adams and Moss, 2008).

The level of Enterobacteriaceae that was found in all samples analysed, corresponds to previous studies in which the level of Enterobacteriaceae on minimally processed fruit was analysed (< 10^1 CFU/g) (Lund, 1992). The aerobic colony and Enterobacteriaceae counts are not used as a microbiological criterion for assessing the microbial quality of minimally processed fruit. These counts are not routinely performed for fresh produce, but can provide useful information regarding spoilage. Levels of faecal indicator organisms including *E. coli* are better for indicating hygienic quality and contamination (Adams and Moss, 2008). *E. coli* was not detected on the TBX medium that was used for screening in any of the samples analysed. It was however identified as the dominant Enterobacteriaceae specie on VRBG.

The only pathogen that was screened for and detected was *Staphylococcus aureus*. This pathogen can cause a variety of diseases in humans. Ingestion of food that is contaminated with staphylococcal enterotoxin can result in severe vomiting and diarrhoea within 1 to 5 hours. This pathogen has been responsible for causing foodborne disease linked to the consumption of minimally processed melon (CAC/RCP, 2011).

The other presumptive pathogen isolates were confirmed to be pathogenic microbes with medical significance. *Klebsiella pneumonia* is responsible for causing a variety of diseases in humans and animals (Brisse, 2006). It is usually associated with healthcare associated infections and meningitis in individuals with a compromised immunity like infants, the ill and elderly (CDC, 2012b). *Klebsiella pneumonia* has recently been reported to cause infection amongst new-born babies in South Africa (Ballot *et al.*, 2012). *Klebsiella pneumonia* carbapenemases (KPCs) show resistance to a broad range of antibiotics. This drug resistance mechanism has rapidly spread in the United States and is being increasingly reported globally and subsequently posing a major health threat around the world (Chen *et al.*, 2012). *Pseudomonas aeruginosa* is a major cause of nosocomial infections and has a great capacity to resist antibiotics (Mesaros *et al.*, 2007).

Some members of the genus *Enterobacter,* specifically *Enterobacter cloacae, Enterobacter asburia* and *Enterobacter aerogenes* are emerging neonatal pathogens that are responsible for various invasive infections that include bacteraemia, respiratory tract infections and endocarditis; all of which are associated with high mortality. It has been shown that some

Citrobacter freundii isolates have obtained virulence traits and have been the cause of diarrhoea and other infections in humans. *C. freundii* has caused sporadic infections and outbreaks.

No *E. coli, Listeria* or *Salmonella* was detected in the whole melons, whole mangos, fresh-cut mangos and melons at the end of shelf life.

The study demonstrates that the minimally processed mangos and melons that were analysed from a fruit processing plant in South Africa are contaminated with bacterial pathogens including *Enterobacter asburiae, Enterobacter cloacae, Citrobacter freundii, Klebsiella pneumonia, Pseudomonas aeruginosa* and this scenario could reflect on the bacteriological quality of other minimally processed products.

2.3 SUMMARY OF RESEARCH FINDINGS

GAUTENG PROVINCE – Microbiological quality (including virological analysis) of parsley from a farm in the Elandsdrift area

Current microbiological specification guidelines by the Department of Health (DoH) for ready-to-eat fresh fruit and vegetables require coliform counts of less than 200/g, yeast and mould counts of < 100 000 (log 4)/g, *Escherichia coli* 0/g and *Salmonella* at 0/25 g. *However, it is important to note that the DoH guidelines provide specifications only for ready-to-eat fresh produce and these values should therefore not be directly used in production or even processing systems. Our current studies are targeted at the development of a realistic specification for harvested and packed produce.* The yeast and mould counts for parsley along the supply chain (including ready to-eat packaged parsley) were mostly higher that the DoH specifications of < 5 log CFU/g.

Although the human pathogens tested for *(Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes* and *Staphylococcus aureus*) were not detected in the parsley samples, the presence of Norovirus GII on the parsley samples from the field and in the packhouse is a potential health risk, since parsley is often consumed raw. It has been reported that sixty percent of foodborne disease outbreaks in the United States, linked to leafy green consumption between 1973 and 2006, was caused by Norovirus (http://www.sciencedaily.com/releases/ 2008/03/080317164356). Since Norovirus GII was not present in the irrigation water, the parsley contamination was probably due to human faecal contamination in the field or the presence of the virus on workers hands in the field and/or packhouse. In WRC report no. 1773/4/12 NoV GII.4, NoV GII.2 were detected in irrigation water and NoVGII.1 was detected on cabbage, NoVGII.9 on tomatoes as well as HAV 1B on tomatoes and lettuce.

GAUTENG PROVINCE – Microbiological quality and safety of lettuce produced on a farm in the Elandsdrift area

Winter Supreme lettuce seedlings (*Lactuca sativa L.* var. *capitata*) were collected every week for 12 weeks including at harvest, in the packhouse (after harvest) and at a retail store. *E. coli* was never detected in lettuce samples and in only 28% of root samples and 14% of soil samples. No *E. coli* O157:H7 was detected either in any of the water sources or the lettuce crops (pre- and postharvest), roots or soil samples tested. *E. coli* O157:H7 was not detected in any of the samples that tested positive for the presence of general *E. coli.*

GAUTENG PROVINCE – Virological analysis of strawberry samples from a farm in the Pyramid district, Tshwane Metropolitan area

Irrigated strawberries were collected monthly for twelve months (September 2012- August 2013. NoV GI, NoV GII and HAV were detected in 4.2% (1/24), 20.8% (5/24) and 4.2% (1/24) of strawberry samples, respectively. Genotypic characterisation of the isolates showed the presence of swine NoV GII.18 in the strawberries. This is the first report of swine NoVs in South Africa, and raises the question of the possibility of zoonotic NoV infection. The detection of NoVs and HAV on the berry fruits highlights the fact that the contamination of fruit from irrigation water occurred in summer when overhead irrigation was used to keep the fruit cool.

GAUTENG PROVINCE & KWAZULU-NATAL PROVINCE – Virological analysis of cabbages from street vendors and two farms (small-scale and commercial)

Cabbage samples were collected from street vendors, a farm near Pretoria in Gauteng and another farm in KwaZulu-Natal. No viruses (NoV GI, NoV GII, and HAV) were detected in any of the cabbage samples collected from four street vendors in Gauteng, a small-scale farm in Gauteng and a farm in KZN. Since no viruses were detected on produce, a direct link between contaminated irrigation water and associated fresh produce could not be established. WRC report no. 1773/4/12 reported the presence NoVGII.1 on one cabbage sample irrigated with water from the Berg River in the Western Cape.

KWAZULU-NATAL PROVINCE – Microbiological quality of fresh produce (broccoli, bell pepper, cabbage, cauliflower, Crisphead lettuce, jam tomatoes, parsley and spinach) from three farms near Cato-Ridge, Camperdown and Richmond respectively

During the twelve month sampling period the coliform and *E. coli* counts of fresh produce (broccoli, lettuce, jam tomatoes, spinach, cabbage, cauliflower, parsley and bell peppers) from all three farms were often higher than the DoH guidelines for fresh produce to be eaten raw. *E. coli* was detected less frequently in the lettuce irrigated with the borehole water when compared to lettuce irrigated with the river water, i.e. 3/10 (33.3%) and 6/10 (60%) of the sampling times respectively. Other pathogens isolated from the fresh produce included *L. monocytogenes*, *Salmonella* spp., *Shigella* spp. and *Campylobacter* spp. It was evident that the fresh produce with the highest microbial contamination was the leafy vegetables, such as lettuce and spinach. *E. coli* was detected less frequently in the lettuce irrigated with the borehole water when compared to lettuce irrigated with the river water, i.e. 3/10 (33.3%) and 6/10 (60%) of the sampling times respectively throughout the season. Crops that are grown closer to the ground are much more susceptible to contamination since they can come into

direct contact with the contaminant through splashed soil or manure during irrigation. Additionally, leafy vegetables have a large leaf surface area, which has been shown to have a high water holding capacity and are therefore at a greater risk of contamination during irrigation.

KWAZULU-NATAL PROVINCE – Microbiological quality and safety of leafy vegetables (spinach and lettuce) from a farm near Cato-Ridge

E. coli counts obtained for spinach and lettuce sampled for a period of three months (August-October 2013) ranged between 3.3-3.7 log CFU/g and 3.8-4.6 log CFU/g. This is unacceptable according to DoH guidelines for ready-to-eat fruit and vegetables. Typical growth was also observed for *Shigella* spp., *Salmonella* spp. and *L. monocytogenes* which could pose a potential health risk, since lettuce is consumed raw and spinach is also often consumed raw in salads.

KWAZULU-NATAL PROVINCE – Microbiological quality and safety of fresh produce (cabbage and lettuce) from two farms near Camperdown

Fresh produce (cabbage and lettuce) samples were collected from two farms in KwaZulu-Natal for a period of three months (July-September 2014). Lettuce generally had a higher microbial load compared to cabbage. Total Heterotrophic bacteria (THB) counts ranged from 3.88-7.21 and 3.08-6.74 log CFU/g for lettuce and cabbage, respectively. *E. coli* counts on cabbage ranged between 0.52-1.12 log CFU/g and on lettuce between zero and 3.2 log CFU/g. There was a significant correlation ($p \leq 0.05$) between the mean counts for irrigation water and vegetables from farm A, and highly significant ($p < 0.01$) for irrigation water and vegetables from farm B. The presence of *L. monocytogenes* and *Salmonella* on some of the lettuce and cabbage samples could pose a potential health risk to the consumer.

LIMPOPO PROVINCE – Microbiological quality and safety of onions and tomatoes from a commercial farm near Polokwane

The aim of this study was to determine the microbiological quality, prevalence of human pathogenic bacteria and to characterise *E. coli* isolates from irrigation water (river, associated water storage dam, water from overhead irrigation pivot point in the onion field and micro-irrigation pipes in the tomato field) as well as fresh produce (onions and tomatoes)**.**

Onions: No *E. coli* was counted for any of the onion samples. Additionally, the number of coliform, fungi and yeast counts on the onion surfaces gradually decreased over the sampling period (from the 3-4 leaf onion growth stage to the market). At this stage the onion samples complied with DoH general microbiological guidelines for ready-to-eat fresh fruit and vegetables. None of the human pathogenic bacteria tested for were isolated from the onion surfaces, i.e. *E. coli* O157:H7, *Salmonella* Typhimurium and *Listeria monocytogenes*. *E. coli* was however isolated from 10% of the onion samples after selective enrichment, indicating its presence at levels lower than the standard detectable limit for plate counts.

Tomatoes: The microbiological counts for the tomatoes were within the limits of the DoH specifications for ready-to-eat fresh fruit and vegetables. None of the human pathogenic bacteria (*E. coli* O157:H7 & *S.* Typhimurium) tested for were present in the tomatoes.

Although water used to irrigate the tomatoes on the farm in Limpopo was contaminated with human enteric viruses including HAV and NoV GI and NoV GII, indicating potential human faecal pollution, none of the viruses were isolated from the tomatoes. In WRC report no. 1773/4/12 NoV GII.4 and NoV GII.2 were detected in irrigation water and NoVGII.9 and HAV 1B on tomatoes from a street vendor in Marabastad.

LIMPOPO PROVINCE – Microbiological quality and safety of peaches from a commercial peach farm near Mookgophong

The microbiological quality of the peaches from the orchard, packhouse entrance and after packaging were mostly acceptable according to DoH guidelines for ready-to-eat fresh fruit and vegetables. *E. coli* was detected on orchard peaches only, *E. coli* O157 (including H7), *Salmonella* spp. and *L. monocytogenes* were not detected in any of the fruit samples. The *E. coli* isolates from peaches were sensitive to all the antibiotics tested (14 in total).

NORTH WEST PROVINCE – Microbiological quality and safety of lettuce from a farm near Skeerpoort

Faecal coliform (FC) counts of up to 3.5 log CFU/g lettuce were observed which is higher than the DoH specifications to be used as guidelines for the microbiological quality of fresh produce. Over the ten month sampling period FC were present during 5 of the 10 (monthly) sampling intervals. *E. coli* was present during 3 of the 10 sampling intervals. *S. aureus* counts were present during 9 of the 10 sampling intervals. *E. coli*, *Bacillus* spp. and *Enterobacter* spp. were the most prevalent bacterial genera isolated from lettuce. *E. coli* was the most prevalent species on lettuce in winter. The sporadic counts noted for bacterial indicators on lettuce coupled with diversity of isolated bacterial species on lettuce suggest contamination from various environmental sources such as contaminated irrigation water, soil, animals as well as human handlers.

NORTH WEST PROVINCE – Microbiological quality and safety of Crisphead lettuce from harvesting to end of shelf life from a farm in the Brits area

According to the suggested coliform limit for fresh produce eaten raw (DoH, 2000) the coliform levels on the lettuce were unacceptably high. A total coliform count of up to 6.4 log CFU/g was observed for the field samples. Although the wash step significantly reduced the aerobic colony (ACC), Intestinal enterococci (IE), coliform and *S. aureus* counts with approximately 1-3 log CFU/g each, the coliform levels were still high at approximately 3-4 log CFU/g in the lettuce packs. Most of the coliform isolated from lettuce surface were also present in the irrigation water and soil. *Enterobacter cowanii, Pantoea agglomerans, Pseudomonas rhodesia, Pseudomonas koreensis* were the predominant coliforms isolated from lettuce at reception and during processing.

However, although *E. coli* was present in the field lettuce samples, no *E. coli* was isolated from lettuce samples at reception in the packhouse, after chilling, washing or throughout storage at 5°C for eight days. *E. coli* and *Listeria* were only detected when coliform numbers exceeded 10000 CFU/g. *Salmonella* was detected when total coliforms exceeded 1000 CFU/g. Although *E. coli* O157:H7 was isolated from water and lettuce samples, they did not carry the toxin genes *stx1*, *stx2*, *ehx* and *eae*.

The predominant IE species were *E. faecium* and *E. avium.* These were present on the lettuce samples throughout the cultivation period. Other species isolated included *E. faecalis, Aeromonas viridians* and *Globicatella saguinis*. Further characterization of antibiotic resistance of *E. faecium* and *E. faecalis* isolated from field samples indicated high resistance of *E. faecalis* to vancomycin, gentamicin, ciprofloxacin and nitrofurantoin compared to *E. faecium*.

S. aureus counts in lettuce packs were < 2 log CFU/g. *Salmonella* spp. were detected on day 8 in the end of shelf life samples only. *Listeria* spp. were present in field samples, at reception and after overnight chilling, but absent after washing and throughout the shelf life study.

NORTH WEST PROVINCE – Microbiological quality and safety of basil from a farm near Brits

Although the microbiological quality of ready-to-eat packaged basil was unacceptable since the coliform, yeast and mould counts for basil were higher that the DoH specifications for ready-to-eat fresh fruit and vegetables, *E. coli* was not detected in any of the basil samples taken along the supply chain (field, packhouse and retailer). *Salmonella* Typhimurium was present in some of the basil samples after harvest, upon arrival at the packhouse, which could pose a potential health risk to the consumer since basil is often consumed raw. However, it is important to note that the pathogen was not detected in any of the field and retail samples.

WESTERN CAPE PROVINCE – Microbiological quality and safety of pears from four commercial farms

The *E. coli* counts were zero for all the pear samples which included pears from the orchard, from the packhouse after harvest and drenching, as well as after controlled atmosphere storage in a communal packhouse. The fungi/yeast, coliform and *E. coli* counts for the pears were acceptable according to the DoH guidelines and were generally lower when compared to most of the other vegetable crops, especially the leafy green vegetables analysed in this study.

It should be kept in mind that pears irrigated with micro-irrigation pipes are mostly not in direct contact with irrigation water. Low hanging fruit in the orchards might however be contaminated with human pathogenic bacteria present in the irrigation water. *E. coli* O157 (including H7) and *Salmonella* spp. were not detected in any of the pear fruit samples, however *Listeria* spp. was detected in one pear sample. The single *E. coli* isolate from pears in the orchard of one of the farms was sensitive to all the antibiotics tested and did not harbour any of the *E. coli* virulence (*stx1, stx2 and eae)* genes tested for.

GAUTENG – Detection of foodborne pathogens and quantification of microflora on various fresh produce obtained from retailer

The microbiological quality of fresh produce (fruit and vegetables) available at four major retailers in Gauteng were evaluated. The total bacterial, mould and yeast counts were determined and it ranged from minimum detectable level (< log 0.3) to the maximum detectable level (> log 8). The microorganism counts vary depending on the climate and season and the natural titres are between 4 and 8 log/ gram fresh produce. Statistical analysis of microorganism counts showed significant differences between some retailers within the commodities. *E. coli* was detected on lettuce, spinach, mushrooms, cucumbers, and rocket. No *Salmonella* spp. or *Listeria* spp. were detected on the 220 samples purchased, but other human pathogens were detected on the commodities such as *Enterococcus faecalis*, *Enterobacter cloacae*, *E. kobei* and *Klebsiella pneumoniae*. *Staphylococcus* spp. were detected, however. *S. aureus* specifically, was not detected in any of the samples.

KWAZULU-NATAL PROVINCE – Microbiological quality of frozen vegetables

This study revealed that heterotrophic microorganisms, coliforms and presumptive human pathogenic bacteria (*L. monocytogenes*, *S.* Typhimurium, *S. aureus*, *Y. enterocolitica*) were present on frozen vegetables produced by two manufacturers (A and B). Heterotrophic microorganisms and pathogens were present on frozen vegetables produced by manufacturers A and B and exceeded log 7 CFU/g in some cases. ICMSF guidelines for frozen vegetables requires ACC counts of < 4 log CFU/g and that *E. coli* should be absent from "in process" samples to verify postblanching sanitation. *E. coli*, *Salmonella* and *L. monocytogenes* should be absent in the end product. *E. coli* and *C. perfringens* were absent from all the vegetable samples. Freezing was shown to cause a reduction in the levels of all microorganisms (HPC, coliforms, *L. monocytogenes*, *S.* Typhimurium, *S. aureus*, *Y. enterocolitica*) over eight weeks. A reduction of HPC of >25% was achieved for all samples. In the case of the pathogenic species, growth was absent in all vegetables after eight weeks of freezing, with the exception of *S. aureus* where a load of log 3.3 CFU/g was detected on green beans produced by manufacturer B. It is evident that commercial freezing and cold storage will not render food products free of pathogenic microorganisms like staphylococci which gain access during processing. This agrees with results previously reported in literature.

GAUTENG – A microbiological survey of fresh-cut fruit and fruit salad from a processing factory

The coliform, yeast and mould counts of the fresh-cut fruit and fruit salad samples were mostly acceptable according to DoH guidelines. Although the coliform counts of 70% in the end of shelf life samples had increased to > 4 log CFU/g, *E. coli* was not isolated from any of the samples collected from the processing factory and at the end of shelf life samples. According to ICMSF guidelines routine testing of fresh-cut fruit for microbiological safety is not recommended. However, testing for *Salmonella* and *L. monocytogenes* can be done when information indicates potential for contamination. *E. coli* O157 (including H7), *Salmonella* spp. or *Listeria* spp. were not detected in any of the fresh fruit salad samples.

GAUTENG – Prevalance of microbial hazards on minimaly processed mangos and melons

In this study, the microbial quality of fresh cut mangos and fresh cut sweet melons from a processing plant in South Africa were investigated during minimal processing. Whole fruit, cut fruit, packaged fruit as well as packaged fruit at the end of shelf life were analysed. The total average plate counts for the mangos were $>10^6$CFU/g and the average Enterobacteriaceae counts were $>10^5$CFU/g. With regards to the pathogens, S. aureus was present while no *E. coli* O157:H7, *Listeria* or *Salmonella* were detected on the minimally processed mangos and melons. The average total plate count for the melons was $>10^9$CFU/g and the average Enterobacteriaceae counts were $>10^8$CFU/ g. Presumptive pathogens were identified as *Citrobacter freundii*, *E. asburiae*, *E. cloacae*, *Klebsiella pneumoniae* and Pseudomonas aeruginosa. This study showed that minimally processed mango and melon is contaminated with potential bacterial pathogens. This may pose a risk to food safety and public health amongst individuals that consume these food products.

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CHAPTER 3

LINKS AND/OR ASSOCIATION BETWEEN MICROBIAL CONTAMINATIONON ON RAW PRODUCE AT HARVEST, STAGES OF PROCESSING AFTER HARVEST AND WATER APPLIED

3.1 INTRODUCTION

The demand for fresh convenience foods and the associated increase in foodborne disease outbreaks linked to increased fresh produce consumption, emphasised the importance of food safety in the supply chain (Jacxens *et al.,* 2009). This motivated governing bodies, the fresh produce industries and food processing companies worldwide to implement control measures to ensure the microbiological safety of all food products. The need for commodity-specific supply-chain management systems and guidelines based on scientific data (including actual natural microbial levels and pathogen presence/absence) has since been identified (Luning *et al.,* 2011).

Subsequently, the microbiological quality as well as contamination and survival potential of human pathogens (*Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella* spp. and *Staphylococcus aureus*) on fresh produce have been a central research question (Erickson, 2010). Fresh produce contamination with human pathogens can happen throughout the pre- and post-harvest stages of production and marketing. Potential sources of contamination at the pre-harvest level are soil, faeces, irrigation water, reconstituted fungicides and insecticides, dust, insects, manure and wild or domestic animals (Althaus *et al.,* 2012). At the post-harvest level the produce could be contaminated by contact with polluted process water, asymptomatic human carriers, harvesting equipment, and transport containers (Althaus *et al.,* 2012).

Due to water scarcity in South Africa, many farmers (rural and commercial) resort to using surface water for irrigation of fresh produce (Nevondo and Cloete, 1999). The microbiological quality of the water is compromised by the presence of informal settlements with inadequate sanitation and access to potable water (Muller *et al.,* 2001; Odjadjare & Okoh, 2010). Further, municipal sewage works are unable to cope with the growing demands for efficient treatment plants due to a lack of capacity, accelerated urbanisation rate and increased population, which contribute to the high level of pollution in South African river systems (Chigor *et al.,* 2013; CSIR, 2010; Teklehaimanot *et al.,* 2014). Microbiological analysis of South African rivers have shown that they pose a risk of transferring enteric bacterial pathogens, viruses and protozoa onto irrigated produce (Gemmell and Schmidt 2012*;* Ijabadeniyi *et al.,* 2011; Olaniran *et al.,* 2009; Okoh *et al*., 2010). Phenotypic (antimicrobial) and genotypic (virulence gene prevalence, DNA fingerprinting) characterisation of *E. coli* (including O157:H7) isolates from different sources, i.e. irrigation water, soil, bird, animal and human faeces have been used to determine if any of them are sources of faecal contamination which could influence the microbiological quality and safety of fresh produce safety (Ateba and Bezuidenhout 2008; Ateba and Mbewe 2011; Aijka *et al*.,

2014; Holvoet *et al*., 2013). Ateba and Mbewe (2014) showed that *E. coli* O157:H7 isolates from a specific farm in South Africa, food products from supermarkets in a particular city or originating from certain water sources, grouped together following cluster analysis of Enterobacterial Repetitive Intergenic Concencus (Eric) PCR profiles. The fact that the human pathogens can survive on fresh produce for extended periods of time, coupled with the presence of an unacceptably high microbial load in irrigation water, represent a potential microbiological hazard that could pose a food safety risk.

An example of the negative impact of foodborne disease outbreaks on a country is the widely published *E. coli* O104:H4 outbreak in the European Union in May 2011 associated with contaminated bean sprout consumption. The outbreak was eventually linked to a German sprout producer after first implicating Spanish cucumbers and tomatoes. The resultant €225 million losses per week of Spanish vegetable producers highlighted the economic impact of these outbreaks and the importance of accurate diagnostic test methods (http://www.bbc.co.uk/news/world-europe-13683270; Anderson, 2011).

In WRC project K5/1773/4 the potential link between microbiological quality of irrigation water and at harvest fresh produce was investigated by testing for indicator and potential pathogenic organisms. This study reported that total coliform and faecal counts for irrigation water and produce at harvest were generally much higher than standards by the World Health Organisation (WHO, 2008), the Department of Water Affairs (DWAF, 1996) and the Department of Health guidelines for ready-to-eat fruit and vegetables (DoH, 2000). It was concluded that up to 30% of microorganisms on the produce surfaces were present as a result of transfer from the irrigation water. Subsequently, WRC project K5/1875/4 was initiated to continue the investigation into the potential link between irrigation water and fresh produce (fruit and vegetables) at as well as after harvest from the farming to the processing stages of production and marketing.

3.2 RESEARCH FINDINGS

3.2.1 GAUTENG PROVINCE – Microbiological quality (including virological analysis) of irrigation water and potential link to the microbiological quality and safety of parsley from a farm in the Elandsdrift area

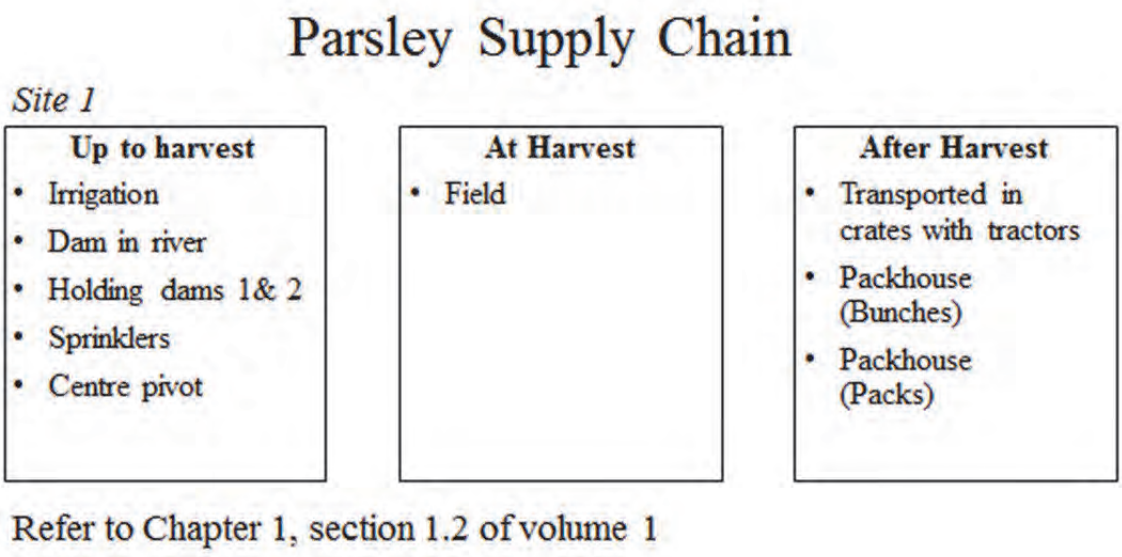
Specific aim

This study aimed to address the lack of scientific knowledge regarding the microbiological quality of fresh herbs at-harvest and after-harvest and the potential impact of irrigation water quality on fresh produce safety.

Experimental procedures

Sampling site

This study was conducted during 2011 on a vegetable/herb production farm in Gauteng using the Crocodile River for irrigation purposes.



Sampling and processing

Irrigation water samples (3 x 1ℓ) were collected from the borehole, Crocodile River, holding dams 1 and 2, sprinklers, centre pivot and nursery and filtrated through 0.45 µm pore size cellulose nitrate membranes. Parsley samples (replicates of 3) were collected during two field trips in 2011 from two fields, from bunches in the packhouse and after packaging. Ten grams of each sample (parsley leaves) were weighed into a sterile polyethylene bag containing 90 mℓ of 0.1% Peptone Water Buffered solution and homogenised.

Microbiological analysis

Decimal dilutions of filter membranes were prepared as described by Standing *et al*. (2013) and plated onto Standard 1 agar (STD 1) and malt extract agar (MEA) to determine total viable bacteria and fungi/yeast numbers. Plates were incubated at 25°C for two (STD 1) and five days (MEA) respectively. The total coliform numbers and presence of *Escherichia coli*, were determined by using a commercially available Colilert test kit according to the manufacturer's instructions (IDEXX Laboratory Inc., One IDEXX Drive, Westbrook, USA, Part no. 98-08877-00). Each of the water samples were enriched for detection of human pathogenic bacteria by incubation at 37 °C for 24 hours, before multiplex PCR analysis.

Samples enriched for determining the presence of *E. coli* (including *E. coli* O157:H7) by incubating the 9 mℓ TSB medium with the filter membranes and homogenised parsley samples at 37°C for 24 hours followed by streaking onto Eosin methylene blue differential medium (EMB) (Merck) for detection of typical *E. coli* colonies. *Salmonella* spp. were detected by enriching each of the water samples in Salmonella enrichment broth for 24 hours at 37°C, followed by streaking onto Xylose lysine deoxycholate (XLD) agar for detection of typical colonies. *Listeria* spp. was enriched in Listeria enrichment broth at 37ºC for 24 hours, where after it was plated onto Listeria agar (all enrichment media were obtained from Oxoid, Johannesburg). All presumptive *Escherichia coli*, *Salmonella* and *Listeria* isolates from selective media were purified by re-streaking onto selective media and identified using PCR analysis and Matrix Assisted Laser Ionisation-Time of Flight (MALDI-TOF).

Determination of *E. coli* O157:H7, *S.* Typhimurium, *Listeria monocytogenes* and *Staphylococcus aureus* presence

DNA was extracted from each of the enriched water and produce samples using the Quick-gDNA miniprep kit (Zymo Research, California, USA). Multiplex PCR analysis was performed using the DNA as template using pathogen specific primers (Cebula *et al*., 1995; Standing *et al*., 2013).

Viral recovery

Irrigation water: Prior to viral recovery the water sample was seeded with a known titre of mengovirus which was used as a process control. Viruses were recovered from the 10 ℓ irrigation water samples using a glass wool adsorption-elution procedure based on the method of Vilaginès *et al*. (1993) as described by Wolfaardt *et al*. (1995) and Vivier *et al*. (2004) and further modified and optimised by Venter (2004). Viruses were further concentrated to a final volume of 10 mℓ using a modification of the polyethylene glycol /sodium chloride (PEG/NaCl) precipitation method originally described by Vilaginès *et al*. (1997) and Minor (1985).

Virus Detection Methods

Nucleic acid extraction: Viral nucleic acid was extracted using two different technologies. Genomic viral nucleic acid was extracted from 1 mℓ of the recovered virus concentrate using the Nuclisens EasyMAG automated magnetic extraction platform (bioMérieux SA, Marcy l'Etoile, France) following the manufacturer's instructions and from a second 1 mℓ of the recovered virus concentrate using the MagNA Pure LC Total Nucleic Acid Isolation Kit (large volume) (Roche Diagnostics GmbH, Mannheim, Germany) in a MagNA Pure LC Robotic instrument (Roche), following the manufacturer's instructions. Purified nucleic acid was aliquoted and stored at -70°C.

Viral amplification and detection

The NoVs, HAV and mengovirus were detected using optimised qualitative rt RT-PCR assays based on TaqMan technology and published primers and probes, namely: HAV (Costafreda *et al*., 2006); NoV GI (da Silva *et al*., 2007; Svraka *et al*., 2007); NoV GII (Kageyama *et al*., 2005; Loisy *et al*., 2005) and mengovirus (Pintó *et al*., 2009). Briefly complementary DNA (cDNA) was prepared using RevertAid™ Premium Reverse Transcriptase (Fermentas Life Sciences, Burlington, Ontario) and amplification was performed using the EXPRESS qPCR Universal Mix (Invitrogen, Carlsbad, CA) in a final volume of 20 µℓ. The reaction was carried out in sealed glass capillaries in a Lightcycler® v2.0 instrument (Roche). An amplification control (AC), which was designed and constructed as part of Project K5/1773//4, was included in each NoV GII reaction. An extraction negative control (nuclease-free water; Promega Corp., Madison, WI), a rt RT-PCR negative control and a *rt* RT-PCR positive control, which comprised of RNA from the target virus, were included in each assay.

Results

Microbiological analysis

Results obtained from the microbiological and virological analysis of water and parsley samples are summarised in Tables 43, 44 and 45.

Water:

Aerobic colony counts (ACC) – The total aerobic counts for all the irrigation water samples (borehole, Crocodile River, holding dams 1 and 2, sprinklers, centre pivot and nursery) ranged between 1.3 log CFU/mℓ (minimum) and 3.1 log CFU/mℓ (maximum) for sampling trip 1 (Table 43). The counts were higher during sampling trip 2 ranging between 2.9 log CFU/mℓ and 4.1 log CFU/mℓ.

Coliform and E. coli – coliform counts using the Colilert test ranged from1550 MPN/100 mℓ to ≥ 2 419 MPN/100 mℓ. The *E. coli* counts were low ranging between 3 MPN/100 mℓ to 81 MPN/100 mℓ.

Fungi and Yeasts **–** the fungi/yeast counts for all seven irrigation water source samples collected during both trips were < 2 log CFU/mℓ.

E. coli O157:H7 – not detected in any of the water samples analysed.

L. monocytogenes – not detected in any of the water samples analysed.

S. Typhimurium **–** not detected in any of the water samples analysed.

Norovirus GI, Norovirus GII and hepatitis A virus – None of the viruses were detected in water samples tested, i.e. Crocodile River, holding dam 1 and 2.

Table 43 Summary of microbiological analysis of water sources used to irrigate parsley

Water sources	Trip 1				Trip 2			
	ACC Log CFU/mℓ	Fungi Log CFU/mℓ	Coliform MPN/100mℓ	*E. coli* MPN /100mℓ	Bacteria	Fungi	Coliform MPN /100mℓ	*E. coli* MPN /100mℓ
Borehole	1.32	2.00	1200.4	113.5	3.24	1.09	*	3.4
Crocodile River	1.95	1.59	*	1.7	3.11	1.35	*	3.7
Holding dam 1	2.36	1.89	2190.7	110.5	4.10	0.88	909.7	36.9
Holding dam 2	2.53	1.71	*	7.8	3.24	1.94	980.4	47.7
Sprinklers	2.71	1.65	*	63.6	2.90	1.36	1245.6	37.1
Center-pivot	2.79	1.82	*	104.7	3.22	0.38	*	58
Nursery	3.06	1.85	*	79.3	3.03	0.98	1018.6	47.7

Parsley:

ACC counts – The total aerobic counts on the parsley varied between 2.8 log CFU/g (minimum) and 6.7 log CFU/g (maximum) (Table 44).

Fungi and Yeasts – minimum of 2.4 log CFU/g and maximum 5.2 log CFU/g, ranging mostly between 4 log CFU/g and 5 log CFU/g.

E. coli 0157:H7 – not detected (Table 45).

L. monocytogenes – not detected.

S. Typhimurium – not detected.

Norovirus GI – not detected.

Norovirus GII – detected on parsley samples from the field and packhouse.

Table 44 Summary of results of microbiological and viral analysis of parsley

Produce	Field trip one		Field trip two	
Parsley	ACC log CFU/g	Fungi log CFU/g	Bacteria log CFU/g	Fungi log CFU/g
Field sample 1	5.81	4.16	5.12	4.63
Field sample 2	6.32	4.60	5.67	4.89
Packhouse (bunches)	6.75	5.18	2.75	2.38
Packhouse (packs)	5.78	4.24	5.20	4.37

Table 45 Human pathogenic bacteria and viral presence/absence in parsley samples

Produce	Human pathogenic bacteria				Viruses			
Parsley	***E. coli* O157:H7**	***S.* Typhimurium**	***L. monocytogenes***	***S. aureus***	**NoVG 1**	**NoVG II**	**HAV**	**Mengo virus**
Field 1	ND	ND	ND	ND	ND	POS	ND	POS
Field 2	ND	ND	ND	ND	ND	POS	ND	POS
Packhouse (bunches)	ND	ND	ND	ND	ND	POS	ND	POS
Packhouse (packs)	ND	ND	ND	ND	ND	POS	ND	POS

ND=None detected; Pos = Positive for pathogen presence

Discussion and conclusion

Current microbiological specification guidelines for ready-to-eat fresh fruit and vegetables require coliform counts of less than 200/g, yeast and mould counts of < 100 000 (log 4)/g, *E. coli* 0/g and *Salmonella* 0/25 g (DoH, 2000). The yeast and mould counts for parsley were mostly higher that the DOH specifications. Although the human pathogenic bacteria tested for were not detected in the parsley samples, the presence of Norovirus GII on the parsley samples from the field and in the packhouse is a potential health risk, since parsley is often consumed raw. Sixty percent of foodborne disease outbreaks linked to leafy green consumption between 1973 and 2006 was caused by Norovirus, ten percent was caused by *Salmonella* spp. and nine percent by *E. coli* (http://www.sciencedaily.com/releases/2008/03/080317164356.htm.)

3.2.2 GAUTENG PROVINCE – Irrigation water quality and potential impact on the microbiological quality and safety of lettuce produced on a commercial farm in the Elandsdrift area

Specific aim

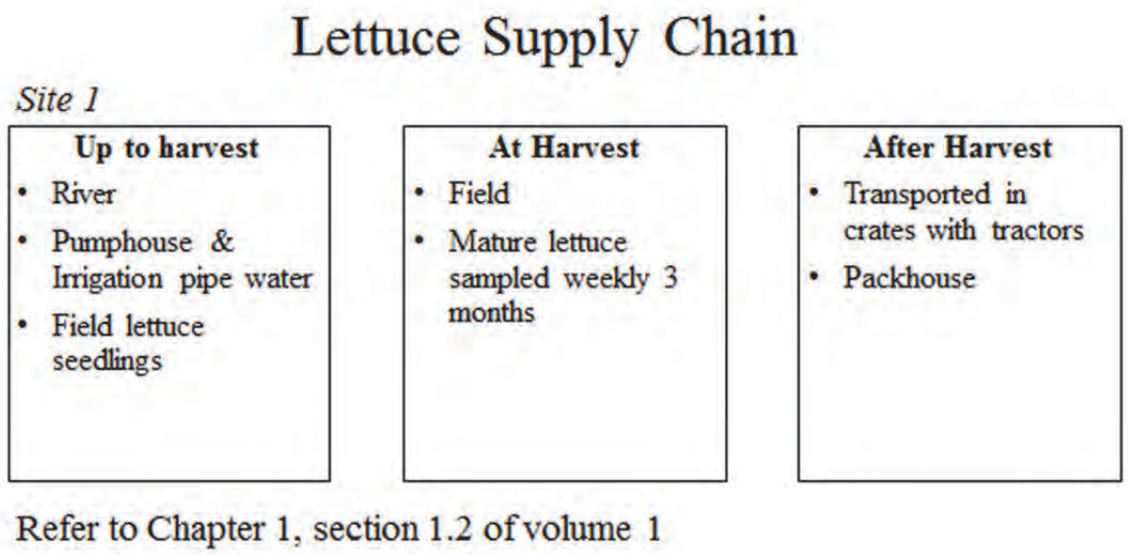
The main objectives were to monitor the microbiological quality of irrigation water (dam, pump house and from irrigation pipes) and the potential impact on the microbiological quality and safety of lettuce.

Experimental procedures

Sampling site

A commercial farm on the banks of the Crocodile River in the Crocodile (West) Marico Water Management Area, in Gauteng was selected. Three different water sources on the chosen farm were tested for quality monitoring purposes: (1) the dam water (fed by river water), (2) the pump house, and (3) water from the irrigation sprinklers on the plot where lettuce samples were collected. River water on the farm is collected in a temporary holding dam which is then pumped and distributed to different areas of the farm through the pump house. Water quality testing at these three points therefore gave an indication of the water

quality at the critical distribution points. The water the crops received was therefore freshly pumped from the main holding dam into which the river flowed.



Sampling and processing

Water:

Triplicate 1ℓ water samples were collected aseptically from each of the water sources every week for a total of 12 weeks. Week 0 was considered as the week before seedlings were transplanted into the fields. Water samples were also collected on the day of harvest to do a comparison with the start of the study. All water samples were membrane filtered through a 0.45µm cellulose nitrate filter (Sartorius, Johannesburg, South Africa). Concentrated cells were then dislodged from the filters through vortexing in 0.1% peptone buffered water (Merck, Pretoria, South Africa) and a serial dilution performed. Cells were plated on Standard 1 Nutrient Agar (Merck, Pretoria, South Africa) supplemented with 1% cyclohexamide (Sigma Aldrich, Johannesburg, South Africa) to quantify the number of viable bacterial cells present. Plates were incubated at 25°C for 48 hours after which colonies were recorded and transformed to log (x + 1) CFU $m\ell^{-1}$.

Triplicate 100 mℓ water samples were collected from each water source in sterile plastic bottles and transported to the laboratory for further analysis within 12 hours. Samples were collected from week 0 until the end of the study. Levels of total coliforms and faecal coliforms were determined using IDEXX Colilert-18® test using the Quanti-Tray®/2000 format (Dehteq, Johannesburg, South Africa) and incubated at 37˚C for 18 hours. Positive wells were converted to MPN/100 mℓ values according to a tabulation of 95% confidence intervals provided by the manufacturer (IDEXX, Maine, USA).

Lettuce: Winter Supreme lettuce seedlings (*Lactuca sativa L.* var. *capitata*) were sprouted on the farm and transplanted into the field. Approximately 15 seedlings were collected with their root systems and rhizospheres 1 week before being transplanted into the field; this sampling day was considered as week 0. Lettuce phyllosphere, root and rhizosphere samples were then collected every week for 12 weeks including at harvest, in the pack house (after harvest) and

at a retail store. Fifteen randomly selected lettuce plants were collected for each sampling period and transported to the laboratory in a cooler-box for further analysis within 12 hours after collection. Lettuce heads were separated aseptically from their root systems and placed in a sterile bag for further analysis. The rhizosphere was then removed from the root systems into a sterile bag by shaking and the roots were then transported in their own sterile bag for further analysis. The outermost leaves of the lettuce heads were aseptically removed and discarded whilst samples were collected from the portion of the head that was considered to be the middle. Samples from 5 separate heads were collected and combined to make up a single replicate of 10 g; three replicates in total were used. Samples from 5 separately collected root systems and rhizospheres were also combined respectively, to make up a single replicate of 10 g; three replicates in total were used for both the root systems and the rhizosphere samples. No root or rhizosphere samples were collected from the pack house or retail samples. The same batch of lettuce was followed from the seedling stage all the way to the retail store with the logistical assistance of the farm manager.

Once the phyllosphere, root and rhizosphere samples were combined into replicates, each replicate was placed in a sterile stomacher bag with 90 mℓ 0.1% peptone buffered water (Merck, South Africa). Phyllosphere and root samples were then stomached for 2 min at 230 rev min^{-1} in a Seward Stomacher 400 Circulator (Seward Ltd., London, UK). Rhizosphere samples were sonicated in an ultrasonic bath for 2 min at room temperature. Serial dilutions were then performed for all of the samples respectively. Cells were plated and incubated as previously discussed. Colonies were recorded and transformed to log (x + 1) CFU g^{-1}.

Nucleic acid extraction and Polymerase Chain Reaction for pathogen detection-
Filters from the water samples and the leaves, roots and soil samples were enriched in Tryptone Soy Broth for 24h at 37°C. DNA was extracted from each sample using a Zymo Quick-gDNA MiniPrep kit (Inqaba Biotech, Johannesburg, South Africa) as per the manufacturer's instructions. *Escherichia coli* and *Escherichia coli* O157:H7 were detected using the primers: Eco1 5'-GACCTCGGTTTAGTTCACAGA-3', Eco 2 5'-CACACGCTGACGCTGACCA-3' (Schippa *et al.*, 2010) and UidAa 5'-GCG AAA ACT GTG GAA TTG GG-3', UidA 5'-TGA TGC TCC ATA ACT TCC TG-3'. A universal 16S reaction was also included in the multiplex PCR reaction as an internal PCR control using the following primers: 27 forward 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492 reverse 5'-ACGGCTACCTTGTTACGAC-3'primers. The PCR amplification was carried out in a GeneAmp 2400 PCR system (Applied Biosystems, Foster City, USA) using Takara EmeraldAmp Max HS PCR Master Mix (Separations, Johannesburg, South Africa). The PCR mixture contained: 25µl EmeraldAmp MAX HS PCR Master Mix; 0.2µM each of Eco 1, Eco 2, UidAa, UidAb, 27 forward and 1492 reverse respectively; 80ng DNA Template; and sterile distilled water. PCR was performed for 30 cycles under the following conditions: 10s at 98˚C, 30 sec at 60˚C and 1 min/kb at 72˚C. The products of the amplification were then analysed by electrophoresis in a 2% (w/v) agarose gel containing 0.01% Ethidium bromide (Merck).

Statistical analysis

Data (log (x + 1) CFU g^{-1} for lettuce leaf, root and soil samples and log (x + 1) CFU $m\ell^{-1}$ for water samples) was analysed using an analysis of variance (ANOVA) with SAS-9.2 software (SAS Institute Inc., Cary, United States of America). Means obtained were compared by the Fishers protected least significant difference (LSD) test at a 5% (P = 0.05) level of significance.

Results

Water analysis

The heterotrophic bacterial counts of the different water sources are summarised in Table 46. Significant interactions occurred between the different water sources and time (F= 35.18; P<0.0001) and therefore this was considered for data analysis. 71% of the water testing periods showed a significant difference between the microbial quality of the dam water compared to the water that eventually reached the crops. On all cases, except one (week 4), the microbial content of the irrigation water was lower than the original source water. 50% of the dam water samples showed a significant decrease in the amount of bacteria present after passing through the pump house and subsequent ozonation. However, 43% of the samples showed no significant difference after ozone treatment with only 7% showing an increase in bacterial content after treatment. The majority of the pump house water samples showed an increase in bacterial content before it reached the irrigation point, however only 50% of these increases were significant. Whereas 83% of the decreases in bacterial content between the pump house and the irrigation point showed significant decreases.

Enumeration of total coliforms and faecal coliform cells by Colilert-18® and PCR

Table 47 shows the total and faecal coliform counts of the water samples collected from the dam, the pump house and at the irrigation point during the study period. All of the faecal coliform values obtained were within South African guidelines for irrigation of crops that are not eaten raw and that are allowed to dry before harvesting (DWAF, 1996). However, according to the WHO the faecal counts obtained showed that the water could be used for unrestricted irrigation of all crops (eaten both raw and cooked) without known ill effects (WHO, 1989). The total coliform values of the source water were all above 1000 counts/100 mℓ except for the reading taken at the start of the study. Four of the 14 test days showed an increase in the number of total coliforms between the dam and the irrigation point; 3 cases showed a decrease and the remaining 7 cases could not be determined, as the limit of the testing method had been reached. Only 4 cases showed an overall increase in the number of faecal coliforms present between the dam and the irrigation point. No conclusive pattern could be identified for the increases/decreases in total and faecal coliform numbers. However, 4 distinct patterns could be seen: 1) gradual decrease in counts between the dam and irrigation point; 2) increase between the dam and pump house followed by a decrease to a value lower than the original starting value; 3) gradual increase in counts between the dam and irrigation point; 4) decrease between the dam and pump house followed by an increase between the pump house and the irrigation point.

Table 46 Number of heterotrophic bacterial plate counts (Log_{10} (x + 1) CFU $m\ell^{-1}$) in dam water, pump-house water and irrigation-pipe water

Time (weeks)	Dam	Pump house	Irrigation pipe
0	3.40273 ijk	2.6735 st	2.8031 rs
1	3.35205 jkl	3.23006 lmno	3.08277 op
2	3.34678 jkl	2.80268 rs	3.13568 op
3	3.2986 klmn	2.63746 tu	2.6822 st
4	3.53999 efghi	3.06941 pq	4.22736 a
5	3.72211 bc	3.19601 mnop	3.57427 defgh
6	3.34199 jklm	3.33303 klm	3.35961 jkl
7	3.48493 fghij	2.94211 qr	3.43659 ghijk
8	3.37834 jk	3.74726 b	3.33508 klm
9	3.65847 bcde	3.33236 klm	3.16098 nop
10	3.58078 cdef	3.60843 bcdef	3.42839 hijk
11	3.73435 b	3.6509 bcde	3.70551 bcd
12	3.5479 efghi	3.66518 bcde	3.29092 klmn
At harvest	3.72185 bc	3.70349 bcd	2.51817 u

All means obtained from three replicates. All means followed by the same letter are not significantly different ($P < 0.05$). An analysis of variance indicated a highly significant difference between the water sources (F=104.30; $P<0.0001$) as well as over time (F= 64.29; $P<0.0001$). As the interactions between the two variables were also highly significantly different (F= 35.18; $P<0.0001$), this relationship was used to analyse data.

Table 47 Total and faecal coliform (MPN/100 mℓ) detection in samples from the dam water, pump-house water and irrigation-pipe water through Colilert-18® analysis

Time (weeks)	Water Sources		
	Dam	Pump house	Irrigation pipe
0	549.73(47.70)	275.33(3.77)	>2419.6(3.05)
1	2202.95(154.20)	1123.17(123.17)	>2419.6(7.87)
2	>2419.6(53.07)	1305.53(22.20)	>2419.6(14.83)
3	>2419.6(34.23)	2046.27(1.33)	>2419.6(7.07)
4	>2419.6(15.57)	1449.93(2.03)	>2419.6(38.57)
5	>2419.6(19.63)	2275.17(4.13)	2202.95(18.83)
6	2130.77(71.07)	>2419.6(78.77)	>2419.6(60.87)
7	1961.80(31.03)	208.13(4.67)	1613.03(26.57)
8	>2419.6(19.70)	>2419.6(23.63)	1769.70(20.73)
9	>2419.6(39.10)	2202.95(27.10)	>2419.6(2.73)
10	>2419.6(54.47)	>2419.6 (56.87)	>2419.6 (3.43)
11	2419.60(148.33)	>2419.6 (194.93)	>2419.6 (277.4)
12	>2419.6(437.13)	>2419.6 (771.17)	>2419.6 (112.23)
At Harvest	>2419.6(109.53)	>2419.6 (160)	>2419.6 (1)

Results obtained for the lettuce phyllosphere, roots and soils are summarised in Table 48.

Table 48 Number of heterotrophic bacterial plate counts (Log_{10} (x + 1) CFU g^{-1}) on lettuce phyllosphere, root surfaces and rhizospheres

Time (weeks)	Phyllosphere	Roots	Rhizosphere
0	5.386^{LMN}	5.459^{KLM}	4.5273^{OP}
1	6.6259^{BCDEF}	6.8922^{BC}	7.3519^{A}
2	6.4486^{CDEF}	7.0447^{AB}	6.9128^{ABC}
3	5.8151^{HIJKL}	7.0598^{AB}	5.8806^{GHIJK}
4	6.1944^{FGHI}	4.9667^{NO}	6.6933^{BCDE}
5	5.5141^{JKLM}	6.8366^{BC}	6.3408^{DEFG}
6	5.9624^{GHIJ}	6.5047^{CDEF}	6.7202^{BCDE}
7	5.6506^{JKL}	6.7911^{BCD}	6.2568^{EFGHI}
8	5.8458^{HIJKL}	6.6665^{BCDEF}	6.2748^{EFGH}
9	5.1179^{MN}	6.6116^{BCDEF}	6.5120^{CDEF}
10	5.4161^{KLMN}	6.6964^{BCDE}	6.3517^{DEFG}
11	5.0837^{MN}	6.8496^{BC}	6.6204^{BCDEF}
12	4.3638^{P}	5.6765^{JKL}	6.7936^{BCD}
At harvest	5.6705^{JKL}	6.6264^{BCDEF}	5.6046^{JKL}
After harvest	4.2869^{P}	N/A	N/A
Retail samples	5.7898^{IJKL}	N/A	N/A

All means obtained from three replicates. All means followed by the same letter are not significantly different ($P < 0.05$).

Polymerase Chain Reaction for general E. coli and E. coli O157:H7 detection

E. coli was detected through PCR in all the water sources (Table 49) at some point throughout the study. On most occasions, a positive correlation existed for *E. coli* between the dam water, the pump house water and the irrigation pipe water. On week 0, the pump hose water showed a positive result for *E. coli* despite the pathogen not being detected in the dam water. The same trend was seen on week 10 when the irrigation pipe water displayed a positive result when the dam water was negative. None of the phyllosphere samples (ranging from seedlings to retail samples) tested over the 12 week period showed the presence of any form of *E. coli*. Root samples from week 2, 6, 7and 9 showed positive results for general *E. coli* but the pathogen was only detected in the soil on week 3 and 6. *E. coli* O157:H7 was not detected in any of the samples that tested positive for the presence of general *E. coli.*

Table 49 Presence/ absence of *E. coli* and *E. coli* O157:H7 in lettuce and water samples

Sample	Time (weeks)													
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**After Harvest**
Phyllosphere	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)
Roots	- (-)	- (-)	+ (-)	- (-)	- (-)	- (-)	+ (-)	+ (-)	- (-)	+ (-)	- (-)	- (-)	- (-)	- (-)
Rhizosphere	- (-)	- (-)	- (-)	+ (-)	- (-)	- (-)	+ (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)
Dam	- (-)	+ (-)	- (-)	+ (-)	+ (-)	+ (-)	+ (-)	+ (-)	+ (-)	+ (-)	- (-)	- (-)	+ (-)	+ (-)
Pump house	+ (-)	+ (-)	- (-)	+ (-)	+ (-)	+ (-)	+ (-)	+ (-)	+ (-)	+ (-)	- (-)	- (-)	+ (-)	+ (-)
Irrigation pipe	- (-)	- (-)	- (-)	+ (-)	+ (-)	+ (-)	+ (-)	+ (-)	+ (-)	+ (-)	+ (-)	- (-)	+ (-)	- (-)

E. coli results shown with *E. coli* O157:H7 results shown in parenthesis

"+" Positive result via PCR

"-" Negative result via PCR

Discussion and conclusion

Due to the potential for crop contamination by pathogenic microorganisms through the application of contaminated water (Castro-Ibáñez *et al*., 2015; Chitarra *et al*., 2014; Holvoet *et al*., 2013) the likelihood of lettuce contamination by general *E. coli* and *E. coli* 0157:H7 was studied during primary production on a working farm in the current study. Ideally, irrigation sources should be properly maintained and monitored for microbiological safety. However, this is extremely difficult in South Africa, as the majority of important agricultural rivers are constantly polluted through the many informal settlements and infrastructure developments along their banks (Ijabadeniyi and Buys, 2012). The shear volumes of water that are used in irrigated agriculture often results in water treatment being unfeasible due to the exuberant costs involved.

In the current study, very notable differences were observed in the quality of the different water sources tested. The heterotrophic plate counts and the Colilert data showed that the water quality improved after being pumped from the dam and passing through the pump house. However in most cases the water quality deteriorated again between the pump house and the irrigation point. The presence of general *E. coli* was also confirmed through PCR analysis. In most cases where *E. coli* was found in the dam water, it was also present in the pump house water and the irrigation water; although this did not occur on all occasions. Changes in water quality within water distribution systems has been reported on numerous occasions as a result of biofilms (Pachepsky *et al.*, 2012). Biofilm formation was not followed in the current study. However, these changes in microbial population can be attributed to either these naturally occurring communities or the introduction of new populations through breaks in the distribution systems. The latter was prevented at all costs on the farm on which the current study was performed so as to minimise overall operating costs.

Although faecal coliforms were detected in the irrigation water that was used for final crop irrigation, the values obtained were still within South African guidelines for irrigation of crops that are not eaten raw and that are allowed to dry before harvesting (DWAF, 1996). Therefore, according to South African guidelines, the water used in the current study was considered unfit for the lettuce crops that were irrigated. A study by Ceuppens *et al*. (2014) showed that the water used for the irrigation of lettuce during primary production on a farm in Brazil was also beyond regulatory levels with 100% of the irrigation water samples tested being positive for total coliforms and 60% positive for *E. coli.* It has been shown previously that the method of irrigation can also greatly contribute towards the degree of crop exposure to pathogens present in the water. In a study by Solomon *et al.* (2002), it was found that more than 90% of spray-irrigated lettuce showed signs of *E. coli* 0157:H7 contamination, whereas less than 20% of surface-irrigated lettuce did. As spray irrigation was used in the current study, the lettuce crops were at a higher risk for contamination. Despite this, none of the lettuce samples collected showed signs of *E. coli* contamination.

Islam *et al*. (2004) found that soil often has high levels of indicator organisms and that these can also contribute towards additional lettuce contamination especially during seasons when large rainfall is experienced, as splash back is often experienced. Studies by Solomon *et al*.

(2002) and Franz *et al*. (2007) showed that *E. coli* O157:H7 from contaminated irrigation water was able to migrate directly from the soil surrounding the root system to the edible portion of lettuce plants through the root conducting tissues. In the current study, 4 of the root samples tested positive for *E. coli*, whereas only two of the soil samples tested positive. Despite these samples showing positive results, *E. coli* did not persist in those environments, as has been demonstrated previously (Islam *et al*., 2004). Islam *et al.* (2004) showed that *E. coli* O157:H7 was able to persist in high numbers for >5 months after the original application of contaminated irrigation water. They also showed that the soil used in their study played a role in the survival of *E. coli* O157:H7 as contamination of crops, without constant direct exposure to *E. coli* O157:H7, still occurred.

The presence of *E. coli* in water used to irrigate lettuce has been observed on numerous occasions, with many studies considering contamination of crops after flooding events. In a study by Castro-Ibáñez *et al*. (2015) it was shown that despite concentrations of *E. coli* in irrigation water being higher than 4 log CFU/100 mℓ after a flood event, only two of the lettuce crops samples showed the presence of *E. coli; E. coli* was not detected again in their study. This same pattern was observed in the current study with no lettuce samples showing the presence of *E. coli*. This is contradictory to many pilot laboratory studies that have shown that *E. coli* can survive on and in lettuce leaves if watered with contaminated water (Islam *et al*., 2004; Solomon *et al*., 2002). These experiments were all under extremely controlled conditions and did not take into account naturally contributing factors such as growth environment, naturally occurring concentrations of pathogen, time of pathogen introduction (Ibekwe *et al*., 2004), solar radiation, environmental temperature (Castro-Ibáñez *et al*., 2015), the type and age of the plant, plant exudates, soil characteristics, and the strain and/or serovar of the pathogen (Chitarra *et al*., 2014), which all play a major determining role in pathogen survival on or in crops.

When taking into account the entire “farm to plate” chain, microbial contamination of crops can occur at any point from vegetable production and harvesting to packing (Islam *et al*., 2004). A study in Pakistan by Ensink *et al*. (2007) showed that contamination did not only occur through the application of poor quality irrigation water, as the authors found that vegetables collected from the local market often showed higher *E. coli* concentrations when compared to the same furrow-irrigated vegetables collected from the fields. Irrigation water quality therefore plays an important role in the quality of crops, but other factors such as post-harvest handling should also be considered.

3.2.3 GAUTENG PROVINCE – Virological analysis and indicator organism levels of irrigation water and linked strawberry samples in the Pyramid district, Tshwane Metropolitan area

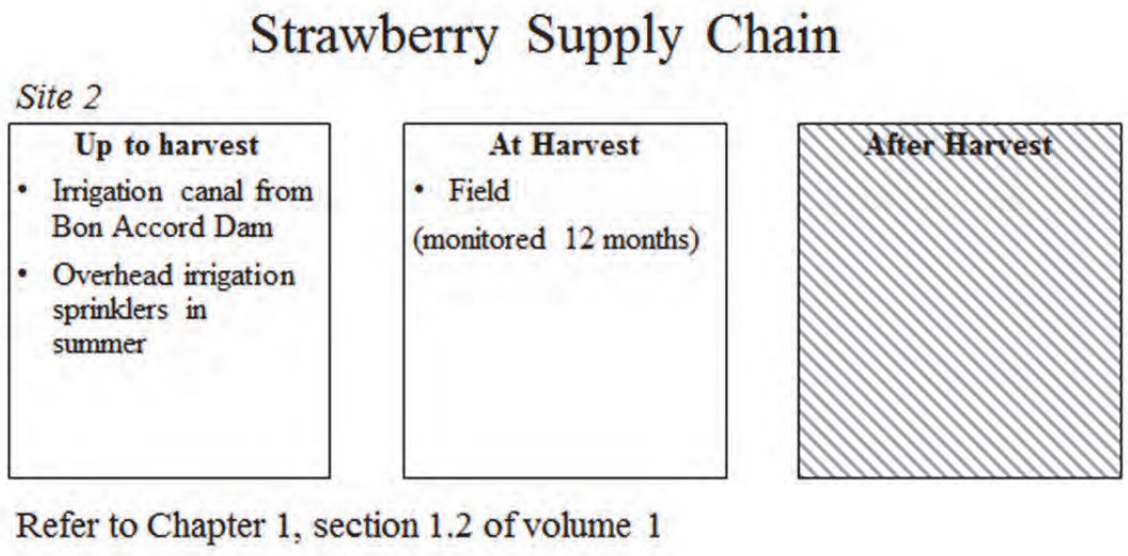
Specific aim

The aims of this study were; i) to assess the quality of surface water used for irrigation of strawberries, ii) to assess the sensitivity of two extraction procedures, iii) to determine the presence of selected enteric viruses (NoVs and HAV) on strawberries and in irrigation water(s) used and, iv) to characterise and determine the phylogenetic relationship of the viruses detected.

Experimental procedures

Sampling site

A small-scale strawberry farmer to the north of Pretoria, Gauteng, was approached for the collection of strawberries, when in season or available, and irrigation water from the irrigation canal flowing from the Bon Accord dam used for the irrigation of the strawberries. It was noted that the farmer used overhead irrigation at the start of the study but changed to drip irrigation soon thereafter when he became aware of preliminary test results



Irrigation water and strawberry sampling

From September 2010 to August 2011 irrigation water samples were collected on a monthly basis from an irrigation canal flowing from the Bon Accord Dam, Pretoria. Water samples were collected in sterile containers (10 ℓ with an additional 1 ℓ) and transported in a cooler bag to the laboratory and stored at 4°C until processed.

To obtain a representative sample size three samples, each comprising of three strawberries, were sampled from different areas of the field and analysed individually. Each strawberry was collected in a separate zip lock bag and gloves were changed between the picking of each

strawberry to prevent possible cross contamination. The samples were placed in a cooler bag and transported to the laboratory and stored at 4°C until processed. The leaves from the base of the strawberries were removed before proceeding with viral recovery.

Analysis for indicator organism

Thermotolerant (faecal) coliforms

Thermotolerant (faecal) coliforms were enumerated from a 100 mℓ aliquot of sampled irrigation water (1 ℓ) using a membrane filtration method (0.45 µm Sartorius Stedim filters). Turbid water samples that could possibly restrain or inhibit the flow of the water through the filter were diluted ten-fold using Modified Scholtens' Broth (MSB). Faecal coliform selective agar plates (m-FC agar plate (55 mm diameter) (Selecta Media, Quantum Biotechnologies [Pty] Ltd, Ferndale, SA) were used onto which the filters were placed and incubated overnight at 44.5°C. Individual blue colonies on the surface of the filter(s) were used to calculate the number of colony forming units (CFU)/100 mℓ present for each water sampled. In cases where individual colonies could not be counted due to the higher number present/over growth, serial ten-fold dilutions of the water were made with PBS (Sigma-Aldrich Co.) and the enumeration process repeated.

Escherichia coli

The process for the enumeration of *E. coli* from the irrigation water samples was identical to the enumeration of thermotolerant (faecal) coliforms with the following differences: i) a 0.45 µm filter from Merck Millipore (Merck) was used, ii) m-ColiBlue24 *E. coli* specific broth (Merck) was used and iii), the plates were incubated overnight at 35°C. The CFU/100 mℓ was calculated by counting the individual blue colonies present. Serial ten-fold dilutions of water were made in cases where individual colonies could not be enumerated.

Viral Recovery from irrigation water and strawberries and virus detection were done as described for parsley in section 3.2.1.

Characterisation of Noroviruses

For the characterisation of NoVs a highly conserved region at the 5'end of the NoV capsid gene was amplified and sequenced. A two-step conventional PCR amplification was performed with published sets of primers. The KAPA Taq Hot Start PCR kit (KAPA Biosystems, Cape Town, SA) with 5 mM dNTP's (Fermentas Life Sciences), 37.5 mM magnesium chloride(KAPA Biosystems), and 5 µℓ of cDNA was used for the first round of amplification. For NoV GI, primer set 1 (Table 50) and for NoV GII primer set 3 (Table 50) with 25 µM and 50 µM forward and reverse primer concentration, respectively, was used. The reaction mix was prepared to a final volume of 20 µℓ. Amplification was performed on a Little Genius thermal cycler (BIOER Technology, Hangzhou, China) using the following cycling conditions; pre-denaturation at 94°C for 10 min, followed by 45 cycles of 94°C for 30 sec, 50°C (NoV GI) or 55°C (NoV GII) for 45 sec, 72°C for 1 min, followed by a final extension step at 72°C for 5 min. A semi-nested PCR protocol was used for the second round of amplification. AmpliTaq® Gold DNA polymerase (0.025 U/µℓ) (Applied Biosystems)

with 5 mM dNTPs (Fermentas Life Sciences), and 1 µℓ of the first round PCR product was used. For NoV GI, primer set 2 (Table 50) and for NoV GII primer set 4 (Table 50) with 5 µM and 25 µM forward and reverse primer concentrations, respectively, was used. Amplification was identical to that of the first round. The PCR products were analysed by 2% agarose gel electrophoresis (SeaKem® LE Agarose, Lonza, Rockland, NY) and visualised by EtBr staining and UV illumination using a 100 bp marker (O'GeneRuler, Fermentas Life Sciences, Burlington, Ontario, Canada) to determine the band size(s).

Table 50 Primer sequences used for genotyping of NoV GI and NoV GII.

Virus	Primer Set	Primer		Reference
		Forward	Reverse	
Norovirus GI	1	QNIF4	G1SKR	Da Silva *et al*. (2007) and Kojima *et al*. (2002)
	2	G1SKF	G1SKR	Kojima *et al*. (2002)
Norovirus GII	3	QNIF2	G2SKR	Loisy *et al*. (2005) and Kojima *et al*. (2002)
	4	G2SKF	G2SKR	Kojima *et al*. (2002)

Cloning of PCR products

Amplified PCR fragments of approximately 300-400 bp were recovered from the gel and concentrated to 10 µℓ using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corp., Irvine, CA). The purified PCR fragments were cloned into a plasmid vector using the CloneJET™ PCR cloning kit (Fermentas Life Sciences) according to the manufacturer's recommendations. Four microliter of the ligation reaction was used to transform the *E. coli* competent cells (10 µℓ) (Lucigen Corp., Middleton, WI). The transformed *E. coli* cells were incubated at 37°C for 1 hour in 900 µℓ recovery media (Lucigen Corp.) under vigorous shaking. A 100 µℓ aliquot of the suspension was plated on LB enriched agar containing 100 µg/mℓ ampicillin (Sigma-Aldrich Co.) and incubated overnight at 37°C. Ten individual colonies were randomly isolated from the plates and screened for the specific insert by PCR amplification. GoTaq® Flexi DNA Polymerase (5u/µℓ) (Promega Corp.) with 2.5 mM dNTP's (Fermentas Life Sciences) and 4 µM pJET 1.2/blunt specific primers according to the manufacturer's recommendations was used. Amplification was performed on a Little Genius thermal cycler (BIOER Technology) using the following cycling conditions; pre-denaturation at 95°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min with a final extension step at 72°C for 10 min. The PCR fragments were analysed by electrophoresis in a 2% agarose gel (SeaKem® LE Agarose) and visualised under UV illumination after staining with EtBr. A 100 bp marker (O'GeneRuler) was used to determine the band size(s).

An additional ten clones were sequenced in those samples where NoV could be typed. The amplification process was repeated for those samples in which an appropriate fragment size could not be obtained with changes made to the nucleic acid concentration (serial tenfold dilution series), the Taq polymerase used for the first and second round amplification steps (KAPA Taq vs. AmpliTaq Gold), the volume of PCR product used for second round amplification (1 vs. 2 µℓ) and the cycling conditions of the PCR assays were adjusted.

Sequencing and sequence analysis of PCR products

Amplified PCR products were purified using the DNA Clean & Concentrator™-25 Kit (Zymo Research Corp.) and eluted in 10 µℓ. The fragments were sequenced based on the Sanger sequencing method using the ABI Prism BigDye® Terminator v3.1 Cycle sequencing Kit (Applied Biosystems) and pJET 1.2/blunt specific primers (3.2 pmol) according to the manufacturer's recommendations. A 2 µℓ aliquot of the purified PCR fragments were used in

each PCR reaction. Amplification was performed on a Little Genius thermal cycler (BIOER Technology) under the following cycling conditions; pre-denaturation at 94°C for 3 min followed by 25 cycles of 94°C for 30 sec, 50°C for 10 sec and 60°C for 4 min. The ABI 3130 automated analyser (Applied Biosystems) was used to analyse the amplified PCR products. Sequencer™ v4.10.1 and MEGA version 5.1 (Tamura *et al*., 2011) was used for the analysis of the sequences. Norovirus reference were strains obtained from (http://0-blast.ncbi.nlm.nih.gov.innopac.up.ac.za/) (Table 51). MAFFT version 6 (http://0-mafft.cbrc.jp.innopac.up.ac.za/alignment/server/index.ht) was used to align the sequences. After manual adjustments of the alignment, phylogenetic analysis was performed with MEGA version 5.1 using the neighbour-joining method. Genotypes were assigned based on the clustering of the individual samples in the phylogenetic tree with a bootstrap support of >70%.

Table 51 Norovirus reference strains used in phylogenetic analysis of field strains.

Genotype	GenBank Accession no.	Strain	Year	Country of Origin
GII.1	HCU07611	Hawaii	1971	United States
GII.2	X81879.1	Melksham	1994	United Kingdom
GII.3	GU980585.1	CBNU1	2006	Korea
GII.3	HM635200.1	Seoul	2009	Korea
GII.4	X76716.1	Bristol	1993	United Kingdom
GII.5	AJ277607.1	Hillingdon	1990	United Kingdom
GII.6	AJ277620.1	Seacroft	1990	United Kingdom
GII.7	AJ277608.1	Leeds	1990	United Kingdom
GII.8	AF195848.1	98-18	1998	Amsterdam
GII.9	AY038599.2	VA97207	1997	United States
GII.10	AF427118.1	Erfurt	2000	Germany
GII.11	AB074893.1	Sw918	1997	Japan
GII.12	AJ277618.1	Wortley	1990	United Kingdom
GII.13	AY113106.1	Fayetteville	1998	United States
GII.14	AY130761.1	M7	1999	United States
GII.15	AY130762.1	J23	1999	United States
GII.16	AY502010.1	Tiffin	1999	United States
GII.17	AY502009.1	CS-E1	2002	United States
GII.18	AY823304.1	OH-QW101	2003	United States
GII.19	AY823306.1	OH-QW170	2003	United States
GII/Unknown	HM560937.1	205	2010	Taiwan

Results

Analysis of indicator organisms

Water:

Coliform and E. coli counts – ranged from 3.49 log to 4.68 log CFU/100 ml and 1.48 log to 3.34 log CFU/100 mℓ, respectively (Table 52).

NoV GI, NoV GII and HAV – Summary of a 12 month survey of the Bon Accord irrigation canal for the presence of indicator organisms of faecal contamination (Table 52).

Table 52 Summary of thermotolerant coliform and *Escherichia coli* counts for irrigation water sources

Sample	Collection Date	Thermotolerant coliforms (log CFU/100 mℓ)*	*Escherichia coli* (log CFU/100 mℓ)*
Irrigation water	2010/09/22	4.00	2.40
Irrigation water	2010/10/25	4.04	2.30
Irrigation water	2010/11/22	4.06	2.40
Irrigation water	2010/12/15	4.68	3.20
Irrigation water	2011/01/21	4.00	3.00
Irrigation water	2011/02/22	3.49	2.67
Irrigation water	2011/03/22	3.98	3.34
Irrigation water	2011/04/21	3.70	2.95
Irrigation water	2011/05/21	4.20	3.04
Irrigation water	2011/06/21	3.78	2.78
Irrigation water	2011/07/26	3.70	3.18
Irrigation water	2011/08/24	3.85	1.48

* CFU/100 mℓ = colony forming units (CFU)/100 mℓ

Viral detection from paired irrigation water and strawberry samples

From September 2010 to August 2011 a total of 24 samples comprising of 72 strawberries in total and 12 irrigation water samples were collected and analysed for the presence of NoV GI, NoV GII and HAV (Table 53). Possible PCR inhibitors were removed by diluting the nucleic acid tenfold prior to amplification. Norovirus GI, NoV GII and HAV were detected in 8.3% (1/12), 41.7% (5/12) and 0% (0/12), respectively, of the irrigation water samples. Similar results were noted in the strawberry samples with an overall detection percentage of 4.2% (1/24), 20.8% (5/24) and 4.2% (1/24) recorded for NoV GI, NoV GII and HAV, respectively.

Table 53 Summary of results for viral analysis using DNA extracted with two different nucleic acid extraction isolation commercially available kits

Sample	Sampling date	Real-time RT-PCR analysis					
		NoV GI		NoV GII		HAV	
		easy MAG	MagNA Pure	easy MAG	MagNA Pure	easy MAG	MagNA Pure
Irrigation water	10.09.22	+	-	-	-	-	-
Strawberry (a)		-	-	-	+	-	-
Strawberry (b)		-	-	-	-	-	-
Strawberry (c)		-	-	-	+	-	-
Irrigation water	10.10.25	-	-	+	+	-	-
Strawberry (a)		-	-	-	-	-	+
Strawberry (b)		-	-	-	-	-	-
Strawberry (c)		-	-	-	-	-	-
Irrigation water	10.11.22	-	-	+	-	-	-
Strawberry (a)		-	-	-	-	-	-
Strawberry (b)		-	-	-	-	-	-
Strawberry (c)		-	-	-	-	-	-
Irrigation water	10.12.15	-	-	-	-	-	-
Strawberry (a)		-	-	-	-	-	-
Strawberry (b)		-	-	-	-	-	-
Strawberry (c)		-	-	-	-	-	-
Irrigation water	11.01.21	-	-	+	+	-	-
Irrigation water	11.02.22	-	-	-	-	-	-
Strawberry (a)		-	-	-	-	-	-
Strawberry (b)		-	-	-	-	-	-
Strawberry (c)		-	-	-	-	-	-
Irrigation water	11.03.22	-	-	-	-	-	-
Irrigation water	11.04.21	-	-	-	-	-	-
Irrigation water	11.05.21	-	-	-	-	-	-

The use of optimised multiplex *rt* RT-PCR assays allowed for the detection of the above and additional viral species (Table 54). Norovirus GI, NoV GII and HAV could be detected in 9.1% (1/11), 36.4% (4/11) and 0% (0/11) in irrigation water samples with the singleplex *rt* RT-PCR assays, respectively. However, with the multiplex *rt* RT-PCR assays NoV GI, NoV GII and HAV was detected in 27.3% (3/11), 27.3% (3/11) and 0% (0/11) of the irrigation water samples, respectively. An additional three NoV GI positive irrigation water samples were detected by the multiplex *rt* RT-PCR assay. Human AstV, HRV and SaV were detected in 36.4% (4/11), 54.5% (6/11) and 9.1% (1/11) of the irrigation waters collected. Mengovirus was consistently detected by both assays.

Table 54 Summary of results from the comparison of singleplex and multiplex *rt* RT-PCR assays for detection of selected enteric viruses

Sample	Sample date	Singleplex rt RT-PCR* assay				Multiplex rt RT-PCR* assays							Total viruses detected
		HAV	NoV GI	NoV GII	PC	HAV	NoV GI	NoV GII	PC	HAstV	HRV	SaV	
Irrigation water	2010.09.22	-	+	-	-	-	-	-	-	-	-	-	1
	2010.10.25	-	-	+	-	-	+	+	-	-	-	-	3
	2010.11.22	-	-	+	-	-	-	-	-	+	-	-	2
	2010.12.15	-	-	-	-	-	+	-	-	+	+	-	3
	2011.02.22	-	-	-	-	-	-	-	-	+	-	-	1
	2011.03.22	-	-	-	+	-	-	-	+	-	-	+	2
	2011.04.21	-	-	-	+	-	-	-	+	-	-	-	-
	2011.05.21	-	-	-	+	-	-	-	+	+	+	-	2
	2011.06.21	-	-	-	+	-	-	-	+	-	-	-	-
	2011.07.26	-	-	+	+	-	+	+	+	-	-	-	3
	2011.08.24	-	-	+	+	-	-	+	+	-	-	-	2

Phylogenetic analysis

The 5’ end of the NoV GII capsid gene (273 bp) was used in the phylogenetic analysis. The NoV GII strains could be genotyped from three of the 10 positive samples, however, no strains from the five NoV GI positive samples could be genotyped (Fig. 27). A neighbour-joining tree using the amino acid sequence alignments of the reference NoV GII strains (Table 9) and unknown NoV strains from this study (Table 55) was drawn (Fig. 27). Phylogenetic analysis could closely match the NoV strain in the irrigation water as swine NoV GII.18 and that of the strawberry as human NoV GII.7. BLAST results of the irrigation water strains found an unidentified NoV GII strain (Table55) recovered from an oyster in Taiwan as the closest match (99% nucleotide sequence identity) (Fig. 27) (Table 56). Analysis of this sequence strain and that of the individual irrigation water sequences closely grouped together with swine NoV GII.18. A pair wise alignment of the amino acid and nucleotide sequences of the Jul/Aug Bon Accord Irrigation Water (BIW) clones, the swine NoV GII.18 and the NoV strain from the oyster (Table 56) revealed an overall 81.8% amino acid and 76.3% nucleotide similarity of the clones to the swine NoV GII.18 strain (Table 56). An overall 99.5% amino acid and a 93.47% nucleotide sequence identity existed between the Jul/Aug BIW clones and that of the oyster NoV GII strain. In the majority of the Jul/Aug BIW clones, insignificant changes were noticed between the NoV GII strain collected in July and that in August 2011 (Fig. 1) (Table 56). Separate groupings of two clones (Gauteng Province (GP) July BIW clone 4 and GP July BIW clone 4) were noticed but these differences were not supported by bootstrap analysis. Similarly, Bon Accord Strawberries (BST) clones 3 and 7 (Fig. 1) grouped together but separate from the other clones, yet the differences noticed were not supported by bootstrap analysis.

Table 55 Summary of genotyping results of NoV GI and NoV GII strains detected in the irrigation water and on strawberries

Sample	Date	Norovirus genogroup	Genotyping
Irrigation water	2010.09.22	GI	Untypable
	2010.10.25	GII	Untypable
	2010.10.25	GI	Untypable
	2010.11.22	GII	Untypable
	2010.12.15	GI	Untypable
	2011.01.21	GII	Untypable
	2011.07.26	GII	GII.18
	2011.07.26	GI	Untypable
	2011.08.24	GII	GII.18
Strawberries	2010.09.22	GII	Untypable
	2010.09.22	GII	Untypable
	2011.06.21	GII	Untypable
	2011.06.21	GII	Untypable
	2011.08.24	GII	GII.7
	2011.08.24	GI	Untypable

Table 56 Pairwise analysis of amino acid and nucleotide sequences of four swine NoVGII.18 strains, with two additional clones of each of the samples respectively, were used to determine the relatedness of the strains to each other and to the two swine NoVGII.18 reference strains (AY823304 and HM560937). A 306 bp region of the NoVGII capsid protein was aligned and used for analysis.

		AY823304	HM560937	Jul 11 BIW				Aug 11 BIW				
		Swine	Oyster	Clone 1	Clone 2	Clone 3	Clone 4	Clone 1	Clone 2	Clone 3	Clone 4	Clone 5
AY823304 Swine			82.18	82.18	82.18	83.17	81.12	82.18	82.18	82.18	79.21	82.18
HM560937 Oyster		77.23		100	100	99	99	100	100	100	97.03	100
Jul 2011 BIW	**Clone 1**	76.24	93.34		100	99	99	100	100	100	97.03	100
	Clone 2	76.57	93.73	99.34		99	99	100	100	100	97.03	100
	Clone 3	76.57	93.4	98.68	99		98.02	99	99	99	96.04	99
	Clone 4	75.91	93.1	99.97	99	98.35		99	99	99	96.04	99
Aug 2011 BIW	**Clone 1**	76.57	93.37	99.67	99.67	99	99.39		100	100	97.03	100
	Clone 2	76.57	93.4	98.69	98.35	98.02	98.35	98.35		100	97.03	100
	Clone 3	76.57	93.73	99.67	99.67	99	99.34	100	98.35		97.03	100
	Clone 4	75.58	93.1	98.35	98.68	98.35	98.02	98.68	97.69	98.68		97.03
	Clone 5	76.24	93.73	99.67	99.34	99	99.34	99.34	99	99.34	98.68	

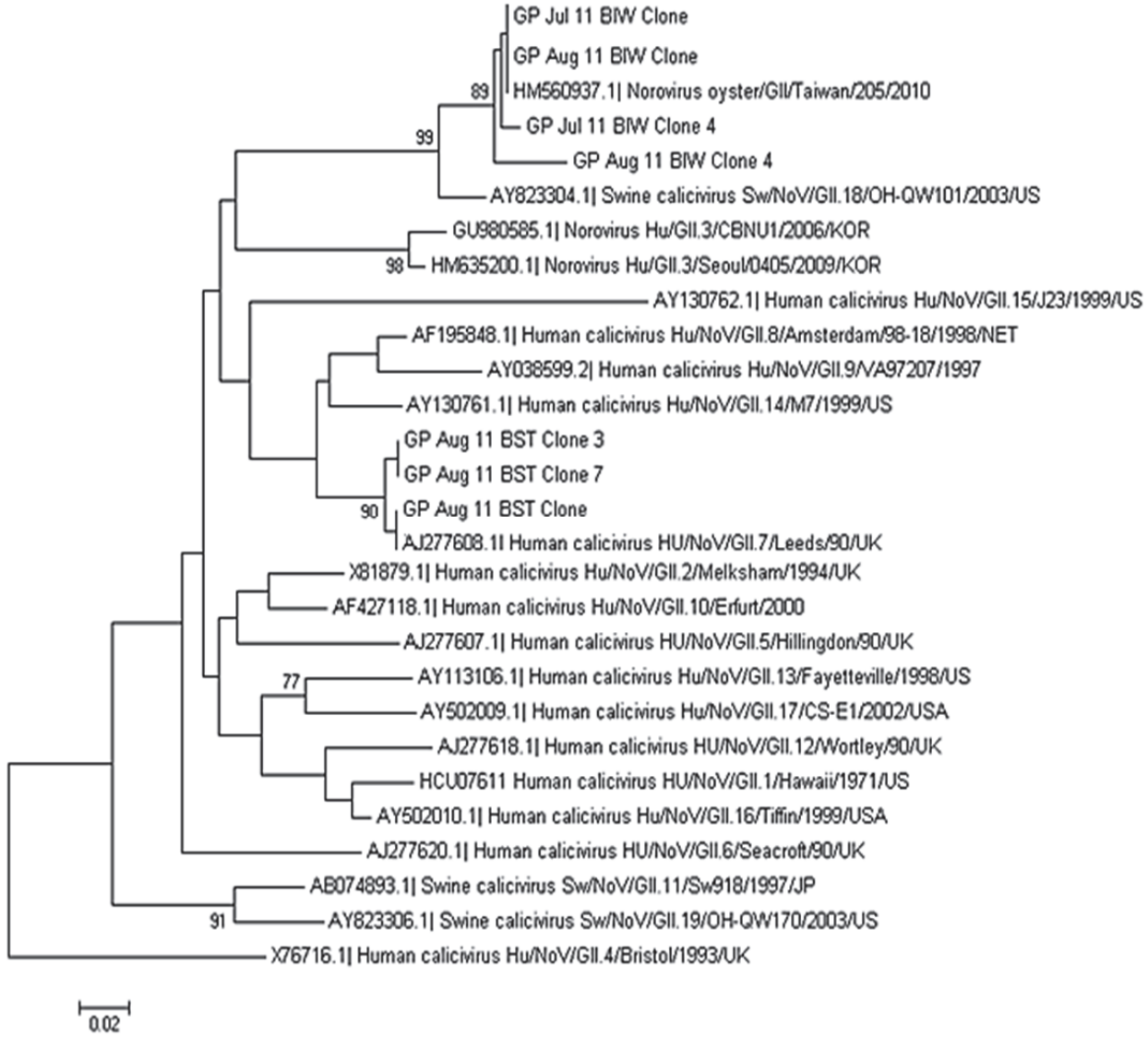


Figure 27 Neighbour-joining phylogenetic tree based on the amino acid sequence of the NoV capsid gene region. The NoV strains from irrigation water and strawberry samples along with reference NoV GII strains were used to draw the tree. Norovirus GII.4 was used to root the tree. Bootstrap percentages are indicated with the bar representing the nucleotide changes. BIW (Bon Accord Irrigation Water) and BST (Bon Accord Strawberries)

Discussion and conclusion

Escherichia coli and thermotolerant coliforms are used as indicator organisms for the presence of enteric viruses in food and water samples because of the cost-effectiveness and effortlessness in their detection. These organisms were used to assess the quality of irrigation water and to correlate their presence with that of selected enteric viruses. The findings indicated that the quality of the water used for irrigation does not meet the standards (thermotolerant coliforms: <1000 colony forming units per 100 mℓ and *E. coli* counts: ≤1 counts/100 mℓ) set by the Department of Water Affairs and Forestry of SA in 1996. The use of bacterial indicator organisms is considered by some as imperfect for the indication of the presence of enteric viruses (Sinclair *et al*., 2009).

A wide range of NoVs have been documented to circulate in SA with NoV GII.4 being the most prevalent strain both in the surface waters and in paediatric patients in SA (Mans *et al*., 2010, 2013). Real-time RT-PCR assays in the form of multi-and singleplex assays were used to determine the presence of NoV GI, GII and HAV in paired irrigation water and strawberry samples. Noro viruses, in particularly NoV GII, were most frequently detected in the irrigation water (Table 55). The multiplex RT-PCR assays applied in this study could detect three additional NoV GI positive samples, however, this could partially be due to the re-extraction of nucleic acid. Additional enteric viruses (HAstV, HRV and SaV) were detected in the irrigation waters used for the strawberries.

Environmental sources could potentially be seen as reservoirs for animal pathogens, more specifically RNA viruses, which could potentially amount to cross-species transmission (Heeney, 2006). The genus *Norovirus* comprises of viruses capable of infecting humans, pigs, cattle and mice species (Patel *et al*., 2009). A risk of zoonotic transmission between human and animal NoV strains could possibly exist. In such an event a novel virus with a new host tropism and increased or decreased pathogenicity, may arise (Almanza *et al*., 2008). Swine NoVs are genetically and antigenically related to the most prevalent human NoVs strain (GII) (Wang *et al*., 2005). Mattison *et al*. (2007) demonstrated the presence of NoV GII.4 human-like strains in pigs whereas Wang *et al*. (2005) identified a potential human-swine recombinant NoV strain that could infect genotobiotic pigs. The possibility for pigs as potential reservoirs for the emergence of new human NoVs strains therefore exists (Wang *et al*., 2005).

During the period Sept 2010 to Aug 2011, 12 water samples were collected of which 42% (5/12) tested positive for NoV GII. Swine NoV GII.18 was genotyped from two irrigation water samples and human NoV GII.7 from a single strawberry sample. Mans *et al*. (2013) had similar success rates in the genotyping of NoVs from surface waters. To our knowledge, swine NoV has not been detected in SA (Mans *et al*., 2010, 2013) and thus makes this the first reported incidence of swine NoV GII.18 in SA. BLAST analysis of this strain indicated a 94% sequence identity with a NoV strain detected in 2010 in Taiwan from an oyster (Fig. 27). Two clones; GP Jul BIW 11 clone 4 and Aug BIW 11 clone 4 grouped separately from the other strains but these differences were not supported by bootstrap values (Fig. 27). Despite these differences, the closest representatives remained swine NoV GII.18 and human NoV GII.7 detected in the water and on the strawberries, respectively. This was the first report on the presence of swine NoV GII.18 in SA.

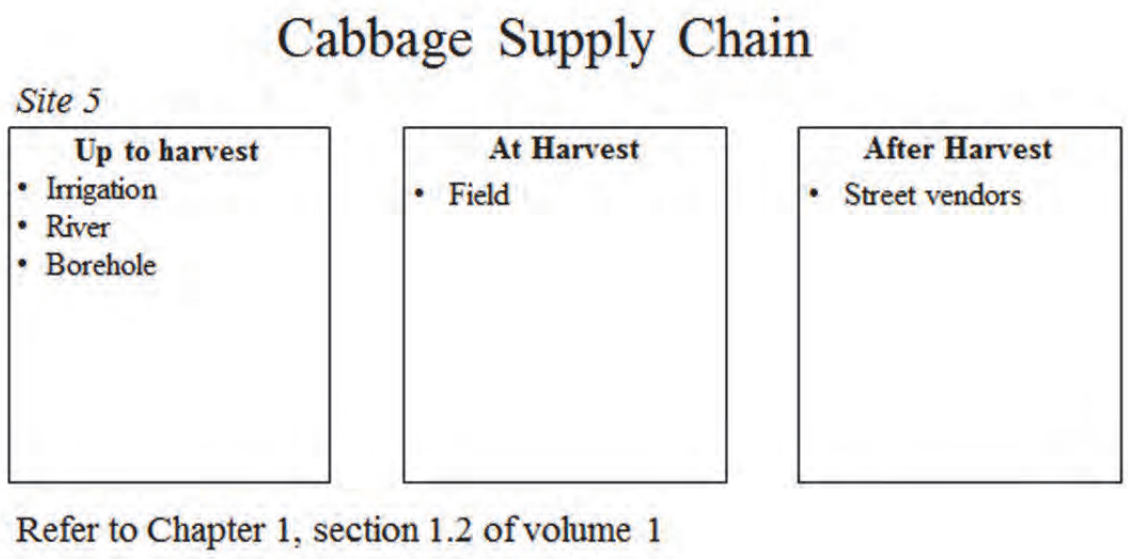
3.2.4 GAUTENG PROVINCE and KWAZULU-NATAL PROVINCE – The extent of virological contamination and indicator organism levels in irrigation water and potential influence on the microbiological quality of cabbages

Specific aim

The objective of this study was to establish if the presence of NoVs and HAV on produce is due to using contaminated irrigation water.

Experimental procedures

Sampling site



Refer to Chapter 1, section 1.2 of volume 1

Irrigation water and cabbage sampling

Water (10 ℓ) and associated cabbages were collected from selected farms in KwaZulu-Natal (river water) and near Pretoria (borehole and river).

Cabbage samples were obtained from street vendors, one farm in Gauteng and another farm in KwaZulu-Natal (KZN). To ensure that each sample was a representative sample, a sample comprised of three individual cabbages bought from the same street vendor or collected from different parts of field. The outer two leaves of the cabbages were removed and discarded and the next three consecutive leaves were sampled and analysed individually.

Microbial indicator analysis

Faecal (thermotolerant) coliforms, *E. coli*, viral analysis including nucleic acid extraction, viral amplification and detection were as described for water used for irrigation of strawberries in the previous section (3.2.3).

Viral analysis was done as described for parsley (section 3.2.1)

Results

Microbial indicator analysis

Faecal (thermotolerant) and *E. coli* were detected in both the river water samples while faecal coliforms were detected at a low level (4 colony forming units (CFU)/ 100 mℓ) in the borehole water (Table 57).

Table 57 Number of colonies counted for faecal coliform and *E. coli.*

Site	Sample	Faecal coliform*	*E. coli* *
KwaZulu-Natal (Farm)	River water	29	24
Pretoria (Farm)	Borehole water	4	0
	River water	70	40

*****Number of coliforms and *E. coli* are expressed as counts/100 mℓ.

Virological analysis

River water samples from the farm near Pretoria tested positive for the process control virus, i.e. mengovirus, and negative for the target viruses, i.e. NoV GI, NoV GII, and HAV. The river water sample from the KZN farm tested positive for HAV and negative for NoV GI and NoV GII (Table 58).

Table 58 Summary of results of viral analysis of irrigation water samples from a farm near Pretoria in Gauteng and a farm in KwaZulu-Natal

Sample site and location	Sample	Real-time RT-PCR			
		NoV GI	NoV GII	HAV	Mengovirus
KwaZulu-Natal (Farm)	River water	Negative	Negative	Positive	Positive
Gauteng (Farm)	Borehole water	Negative	Negative	Negative	Positive
	River water	Negative	Negative	Negative	Positive

From June 2013 to September 2013, five fresh produce samples (cabbage) were collected from four street vendors (4), a small-scale farm in Gauteng and a farm in KZN. No potentially pathogenic human enteric viruses, namely HAV, NoV GII and NoV GI, were detected from any of the cabbage samples (Table 59).

Table 59 Summary of results of the viral analysis of cabbage samples from four street vendors, a farm near Pretoria in Gauteng and a farm in KwaZulu-Natal

Sample	Real time RT-PCR			
	NoV GI	NoV GII	HAV	Mengovirus
Street Vendors (1-4)				
1.1	Negative	Negative	Negative	Positive
1.2	Negative	Negative	Negative	Positive
1.3	Negative	Negative	Negative	Positive
2.1	Negative	Negative	Negative	Positive
2.2	Negative	Negative	Negative	Positive
2.3	Negative	Negative	Negative	Positive
3.1	Negative	Negative	Negative	Positive
3.2	Negative	Negative	Negative	Positive
3.3	Negative	Negative	Negative	Positive
4.1	Negative	Negative	Negative	Positive
4.2	Negative	Negative	Negative	Positive
4.3	Negative	Negative	Negative	Positive
Farm 1 Gauteng				
1.1	Negative	Negative	Negative	Positive
1.2	Negative	Negative	Negative	Positive
1.3	Negative	Negative	Negative	Positive
Farm 2 KwaZulu-Natal				
1.1	Negative	Negative	Negative	Positive
1.2	Negative	Negative	Negative	Positive
1.3	Negative	Negative	Negative	Positive

Hepatitis A virus characterisation

BLAST analysis of a ~ 390 bp region of the VP1/2B junction of the HAV strain detected in the irrigation water (KZN) showed a high percentage genetic relatedness, i.e. 82% nucleotide sequence identity, to a Tunisian strain (Tun40-03) belonging to sub-genotype IA (Gharbi-Khelifi *et al.*, 2006). However, the neighbour-joining phylogenetic tree based on the VP1/2B region sequence grouped the strain within genotype V (Fig. 28).

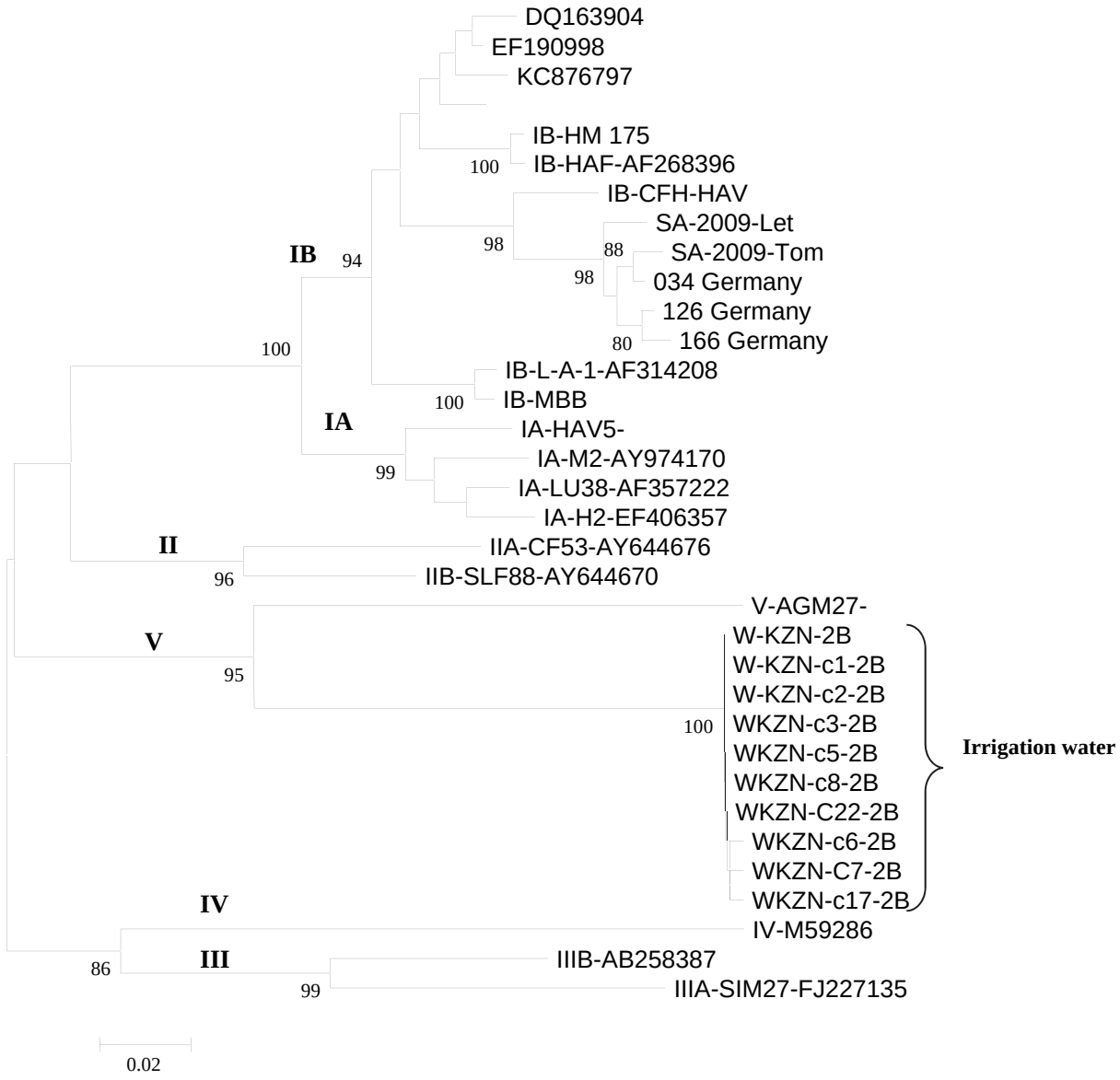


Figure 28 Neighbour-joining phylogenetic tree based on the ~ 390 base-pair VP1/2B region of the HAV strain detected in the irrigation water sample from the farm in KwaZulu-Natal and HAV reference strains. Bootstrap percentages are indicated.

Discussion and conclusion

The *E. coli* counts in the irrigation sample were generally low < 40 CFU/100 mℓ, which makes it suitable for irrigation of crops not to be eaten raw (DWAF, 1996). The presence of HAV implicated potential faecal contamination of human origin. Genetic analyses based on phylogenetic analysis of the 168-nucleotide fragment encompassing the VP1/2A junction, and the full length VP1, VP2 and VP3 of 152 strains of HAV from various parts of the world, have allowed characterisation of the HAV strains and grouping into different genotypes and subgenotypes (Robertson *et al*., 1992; Costa-Mattioli *et al*., 2003). Thus, HAV was classified into genotypes I, II, III and VII that were associated with humans, and IV-VI that were isolated from simian species that had developed a hepatitis A-like illness (Costa-Mattioli *et al*., 2003). An HAV genotype can be defined as a group of viruses with > 85% nucleotide sequence identity (Robertson *et al*., 1992; Costa-Mattioli *et al*., 2003). However, BLAST

analysis showed the strain to belong to genotype IA with genetic relatedness of 82% (nucleotide sequence identity) with a strain that was detected in Tunisia (Tun40-03) (Gharbi-Khelifi *et al*., 2006). The neighbour-joining phylogenetic tree based on the conserved ~ 390 bp VP1/2B region grouped the strain within genotype V with a bootstrap value of 95% (Fig. 2). The latter finding suggests the strain to be associated with non-human primates, i.e. simian species (Costa-Mattioli *et al*., 2003). Genotype V strain (AGM-27) was first described after isolation from an African green monkey from Kenya **(**Andzhaparidze *et al*., 1987). Another study with a report of genotype V strain is that conducted in India, in which simian HAV was isolated from a faecal sample from a rhesus monkey in 1995 (Aranakalle and Ramakrishnan, 2009). This was an interesting result as Taylor *et al.* (1997) showed subtype IB to be the predominant strain in SA.

To date no viruses were detected in the limited number of samples collected post-harvest which were analysed for NoV GI, NoV GII, and HAV. From the current data it is evident that the viral recovery and concentration and the nucleic acid extraction procedures were valid. The real-time RT-PCR assays applied, for the detection of the viruses, had internal amplification controls which confirmed the success of the amplification and therefore the detection assays were valid. the lack of viruses present on the fresh produce could thus be ascribed to: i) no contamination pre-harvest or during harvest, and ii) no contamination during post-harvest food handling. Although 5 samples, comprising 3 leaves each from 15 cabbages, were analysed individually for 4 viruses, this nevertheless represents a small sample size collected at a single time point from the farm or street vendor. Except for detecting Norovirus G1 in one of the cabbage samples, none of the viruses were detected in any of the other cabbage samples.

3.2.5 KWAZULU-NATAL – Microbiological quality of water used to irrigate raw produce (broccoli, cabbage, Chinese cabbage, red cabbage, cauliflower, Crisphead lettuce, jam tomatoes, parsley and spinach) from three farms near Cato Ridge, Camperdown and Richmond respectively

Specific aim

This study investigated the potential link between the irrigation water and fresh produce microbiological quality and safety from three farms in KwaZulu-Natal (KZN).

Experimental procedures

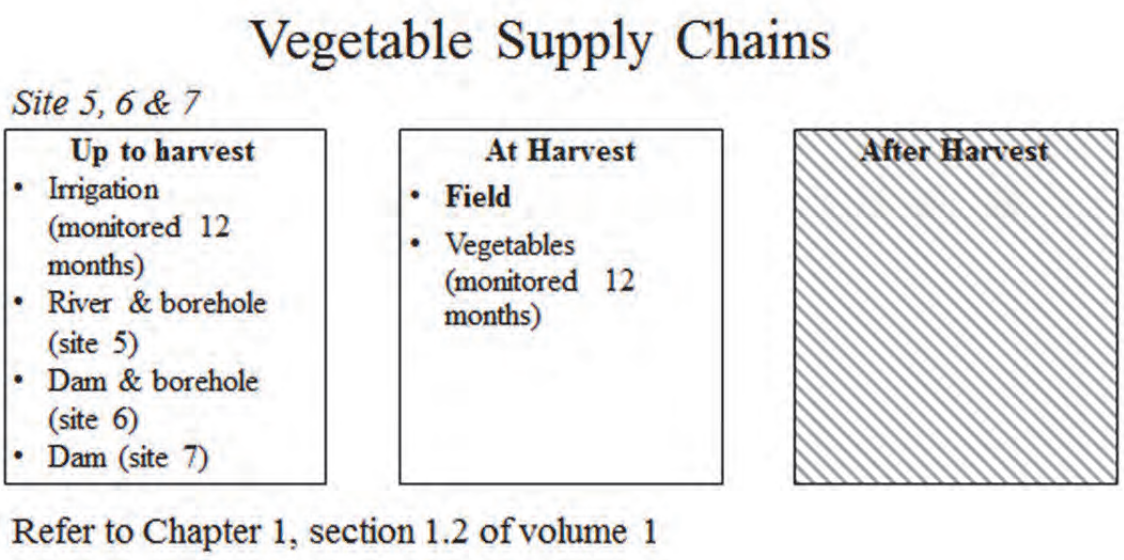
Description of sampling sites

Three different farms, designated A, B, C were used in this study. Farm A (130 hectares) is located in Camperdown. Crops planted are broccoli, spinach, jam tomatoes, Crisphead lettuce, cauliflower and cabbage. Crops are not washed and packed on site. The farm has two sources of irrigation water, namely river (A1) and borehole water (A2), and used weekly to irrigate the plants.

Farm B (60 hectares) is located in Cato Ridge. Crops planted are broccoli, spinach, lettuce, cauliflower, Chinese cabbage, parsley and red cabbage, and these crops are irrigated weekly.

Crops are washed and packed on site. The farm's source of irrigation water is a mixture of borehole and dam water (B).

Farm C (360 hectares) is located in Richmond. Crops planted are oranges, cabbage (planted in winter) and jam tomatoes (planted in summer). Crops are washed and packed on site. The farm's source of irrigation water is dam water (C).



Sample collection and processing

Irrigation water and fruit and vegetables (crops selected were determined by seasonal availability) were collected monthly from the farms for a period of 1 year from July 2009 to June 2010. One litre water samples (x 3) were collected from the respective irrigation sources on the farms. Serial dilutions were prepared from each water sample using sterile distilled water. Hundred and fifty millilitres of the appropriate dilutions were filtered through a membrane filter (0.45 µm). Individual produce samples were prepared by adding ten grams (fresh weight) of the edible portions of vegetables/ fruit to 90 mℓ 0.1% peptone water and homogenised using a blender. Serial tenfold dilutions were then prepared. Selective media used for growth of coliforms (*E. coli*, *Listeria monocytogenes*, *Salmonella* spp. and *Shigella* spp.)were Coliform-chromo agar, Agar Listeria Ottaviani and Agosti (ALOA) Agar respectively.

Results

Microbiological analysis of the irrigation water

The microbial load of the irrigation water sources from farm A and B increased during the summer months (October 2009 to March 2010). Overall, the microbial numbers in the dam water from farm C were low.

Farm A (river A1) – the coliform counts ranged from 2000 (3.40 log) CFU/100 mℓ to 7000 (3.84 log) CFU/100 mℓ from July 2009-June 2010. *E. coli* was present during July and August 2009 only at values below 1000 CFU/100 mℓ (Fig. 29a).

Farm A (borehole A2) – the coliform counts ranged from zero to 5000 (3.70 log) CFU/100 mℓ over the entire twelve month sampling period. *E. coli* counts ranged from zero for all the water sampling periods, except once during October 2009 when the count was 6000 (3.78 log) CFU/100 mℓ(Fig. 29b).

Farm B (mixture borehole and dam water B) – the coliform counts ranged from 3 (0.47 log) CFU/ 100 mℓ to 9 000 (3.95 log) CFU/100 mℓ over the entire twelve month sampling period. *E. coli* counts were zero Fig. 30a).

Farm C (dam water C) – Coliform bacteria were counted only once at < 740 (2.87 log) CFU/100 mℓ and *E. coli* counts were zero during the twelve month sampling period (Fig. 30b).

L. monocytogenes – Typical growth on selective media was not observed for all the water source samples, except once during October 2009 for the borehole water sample [farm A(a)].

Salmonella spp. – Typical growth on selective media were observed once for each of the samples i.e. river water [farm A (a)], borehole water [farm A (b)] as well as mixed dam and borehole water (B) during the twelve month sampling period.
Shigella spp. **–** Typical growth was observed for all the water samples, except for sample C (dam water).

Campylobacter – Presumptive *Campylobacter* spp. was only detected in water sample C (dam water. The population increased when the irrigation water was not in use and the dam was allowed to be stagnate. This could be due to survival and growth of the *Campylobacter* spp. which is a microaerophile in stagnant water with limited oxygen levels (Moore *et al*., 2006).

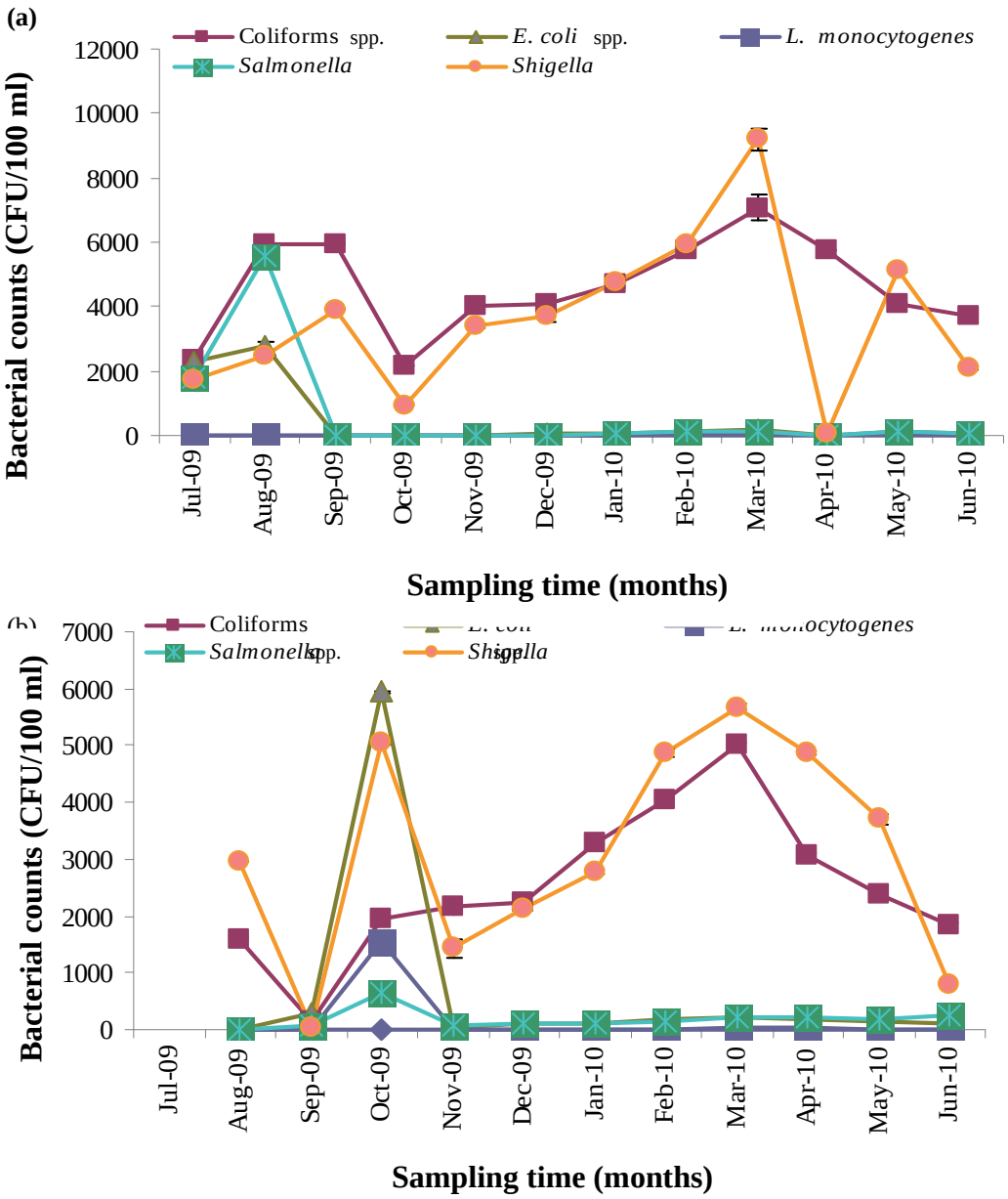


Figure 29 The microbial quality of irrigation water collected from farm A (river water) (a) and A (borehole water) (b), over a one-year period

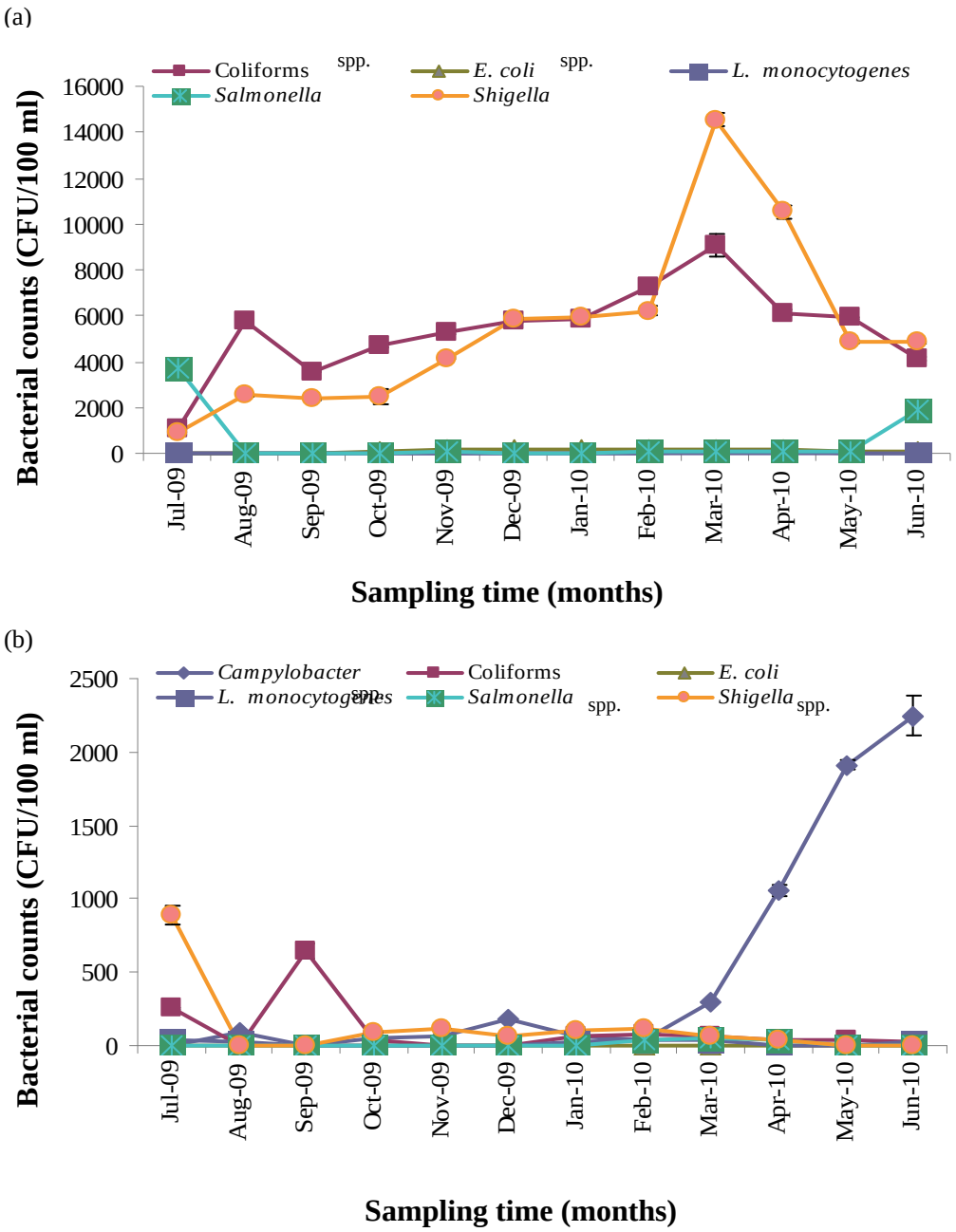


Figure 30 The microbial quality of irrigation water collected from farm B (a) and C (b), over a one-year period

Fresh produce from farm A irrigated with river water

Farm A fresh produce included broccoli, lettuce, jam tomatoes and spinach:

Coliforms – Coliform counts of broccoli, lettuce and spinach were unacceptable according to the DoH guidelines for ready-to eat fresh produce. Jam tomato coliform counts were acceptable for 50% of the samples analysed (Table 60).

E. coli – most abundant in Crisphead lettuce (counts ranged between zero and 5.45 log CFU/g) and spinach (counts ranged between 3.7 log CFU/g and 5.9 log CFU/g). *E. coli* in lettuce irrigated with river water was detected 6/10 (60%) sampling times (Table 60).

L. monocytogenes – least abundant in the fresh produce tested, with the most being recorded at 5.51 log CFU/g for Crisphead lettuce (January 2010) (Table 60).

Salmonella was detected frequently in the edible portion of the spinach and Crisphead lettuce plants (Table 60).

Campylobacter spp., coliforms and *Shigella* spp. were the most abundant on fresh produce (Table 60).

It was apparent that the fresh produce with the highest microbial contamination was the leafy vegetables, such as lettuce and spinach, throughout the season.

Table 60 The monthly variation in presumptive bacterial counts (in CFU/g) on broccoli (fields 1 and 2), Crisphead lettuce, jam tomatoes and spinach collected from farm A irrigated with river water over a one-year period.

Produce/ Sampling Date	SH	SAL	LM	EC	TC	CAMP
Broccoli/field 1						
Jul-09	6.43	N	N	N	3.71	4.50
Aug-09	4.49	N	N	N	4.12	3.82
Sep-09	ND	ND	ND	ND	ND	ND
Oct-09	N	N	N	N	4.30	4.03
Nov-09 - Jun-10	ND	ND	ND	ND	ND	ND
Broccoli/field 2						
Jul-09	5.63	N	N	3.51	3.51	4.49
Aug-09	3.50	N	N	N	3.77	4.85
Sep-09	5.50	N	N	N	4.96	4.91
Oct-09	3.64	N	N	N	3.50	5.31
Nov-09 & Dec-09	ND	ND	ND	ND	ND	ND
Jan-10	3.67	N	N	N	4.31	4.54
Feb-10	ND	ND	ND	ND	ND	ND
Mar-10	3.87	3.51	N	N	4.43	4.68
Apr-10 - Jun-10	ND	ND	ND	ND	ND	ND
Crisphead lettuce						
Jul-09	5.57	N	3.61	3.70	3.82	5.66
Aug-09	3.79	N	N	N	3.62	4.26
Sep-09	3.88	N	N	N	4.84	4.62
Oct-09	N	N	N	N	4.37	4.01
Nov-09 - 7 Dec-09	ND	ND	ND	ND	ND	ND
Jan-10	5.94	3.83	4.05	5.51	5.32	4.32
Feb-10	4.47	N	N	N	4.95	4.51
Mar-10	3.98	3.55	N	5.42	5.22	4.52
Apr-10	5.83	3.67	3.93	4.47	5.24	4.33

Produce/ Sampling Date	SH	SAL	LM	EC	TC	CAMP
May-10	5.73	3.71	3.83	5.45	6.09	4.29
Jun-10	5.47	3.82	N	5.37	3.99	4.44
Jam tomatoes						
Jul-09	5.01	N	N	N	N	4.20
Aug-09	3.49	N	N	N	N	3.90
Sep-09	N	N	N	N	4.34	4.54
Oct-09 - Mar-2010	ND	ND	ND	ND	ND	ND
Apr-10	N	N	N	N	4.40	4.64
Jun-10 - May-10	N	N	N	N	4.10	4.46
Jun-10	ND	ND	ND	ND	ND	ND
Spinach						
Jul-09 - Sep-09	ND	ND	ND	ND	ND	ND
Oct-09	4.79	N	N	N	5.96	6.03
Nov-09	3.55	N	N	N	3.70	3.91
Dec-09	3.95	3.51	3.51	4.31	5.27	5.52
Jan-10	5.32	4.55	3.84	4.63	5.68	4.31
Feb-10	3.93	4.18	N	N	3.85	3.96
Mar-10	4.40	4.35	N	4.99	4.19	4.16
Apr-10	3.86	4.13	N	N	3.78	3.89
May-10	4.35	4.41	N	5.01	4.08	4.10
Jun-10	5.13	4.92	3.73	4.75	5.37	4.56

SH = *Shigella* spp.; SAL = *Salmonella* spp.; LM = *L. monocytogenes*; EC = *E. coli*; TC= Total coliforms; CAMP = *Campylobacter* spp.; ND = Not determined as the fresh produce was not present at the time of sample collection; N = bacterial species was not detected.

Fresh produce from farm A irrigated with borehole water

Farm A fresh produce included broccoli, cabbage, Crisphead lettuce, spinach and cauliflower:

Coliforms **–** Coliform counts of broccoli, lettuce and cauliflower were unacceptable according to the DoH guidelines for ready-to-eat fresh produce (Table 61).

E. coli – most abundant in the Crisphead lettuce samples (counts ranged between zero to 5.97 log CFU/g) and was detected in 3/9 (33.33%) of the sampling times. *E. coli* was detected less frequently in the lettuce irrigated with the borehole water than lettuce irrigated with river water, which was detected in 6/10 (60%) of the sampling times (Table 61).

L. monocytogenes – least abundant in the fresh produce tested, with the highest being recorded at 5.97 log CFU/g in Crisphead lettuce (January 2010) which is similar to the results obtained for lettuce irrigated with river water (5.51 log presumptive *L. monocytogenes* CFU/g) (Table 61).

Salmonella was detected in lettuce 3/9 (33.33%) of the sampling times in the edible portion of the Crisphead lettuce plants. The microbial analysis of the fresh produce collected from farm A irrigated with borehole water showed that the presumptive *Shigella* spp.,

Campylobacter spp. and coliform counts were found abundantly throughout the seasons (Table 61).

Table 61 The monthly variation in presumptive bacterial counts (in CFU/g) on fresh produce irrigated with borehole water collected from farm A over a one-year period

Produce/ Sampling Date	SH	SAL	LM	EC	TC	CAMP
Broccoli						
Jul-09	ND	ND	ND	ND	ND	ND
Aug-09	3.48	N	N	N	4.02	4.77
Sep-09	4.00	N	N	N	4.54	4.34
Oct-09 - Dec-09	ND	ND	ND	ND	ND	ND
Jan-10	6.18	N	N	N	4.70	4.29
Feb-09 - May-10	ND	ND	ND	ND	ND	ND
Jun-10	3.74	N	N	N	3.75	4.39
Cabbage						
Jul-09	4.54	N	N	N	N	4.19
Aug-09	5.00	N	N	N	3.72	4.81
Sep -09	4.46	4.16	N	N	4.91	6.25
Oct-09	N	N	N	4.62	N	4.92
Nov-09 - Apr-10	ND	ND	ND	ND	ND	ND
May-10	N	N	N	N	N	4.06
Jun-10	4.47	N	N	N	N	4.31
Crisphead lettuce						
Jul-09	3.96	N	N	N	N	5.83
Aug-09	4.92	N	N	N	4.47	4.85
Sep -09	4.81	N	N	N	4.54	4.86
Oct-09	4.52	N	N	N	5.91	5.84
Nov-09	N	N	N	N	4.96	3.61
Dec-09	3.49	3.54	3.50	3.56	5.56	4.68
Jan-10	5.32	4.41	3.59	5.97	5.68	4.47
Feb-10 & Mar-10	ND	ND	ND	ND	ND	ND
Apr-10	3.48	N	3.48	N	5.02	3.69
May-10	3.48	4.31	3.48	5.52	5.20	4.58
Jun-10	ND	ND	ND	ND	ND	ND
Spinach						
Jul-09	3.72	N	N	N	N	6.41
Aug-09 - Sep-09	ND	ND	ND	ND	ND	ND
Oct-09	N	N	N	N	3.85	4.08
Nov-09 - Jun-10	ND	ND	ND	ND	ND	ND

Produce/ Sampling Date	SH	SAL	LM	EC	TC	CAMP
Cauliflower						
Jul-09	ND	ND	ND	ND	ND	ND
Aug	5.50	N	N	N	6.05	4.97
Sep-09 - Jun-10	ND	ND	ND	ND	ND	ND

SH = *Shigella* spp.; SAL = *Salmonella* spp.; LM = *L. monocytogenes*; EC = *E. coli*; TC= Total coliforms; CAMP = *Campylobacter* spp.; ND = Not determined, as the fresh produce was not present at the time of sample collection; N = bacterial species was not detected.

Fresh produce from farm B

Farm B fresh produce included: cabbage, Chinese cabbage, Red cabbage, broccoli, Crisphead lettuce, spinach and parsley.

Coliforms **–** Coliform counts of the produce were mostly unacceptable according to the DoH guidelines for ready-to eat fresh produce (Table 62).

E. coli **–** was not detected in broccoli, cabbage, Chinese cabbage and red cabbage collected from farm B, throughout the study. In Crisphead lettuce, *E. coli* was detected at 3.54 log CFU/g during winter (June 2010). The *E. coli* incidence was 1/7 (14.28%), which is much lower than the lettuce from Farm A irrigated with river as well as borehole water, which was 60% and 33.33% respectively (Table 62).

L. monocytogenes – incidence in Red cabbage was 1/4 (25%) of the sampling times and in Crisphead lettuce 1/7 (14.28) of the sampling times. None was detected in any of the other produce samples.

Salmonella was detected in spinach 4/4 (100%) of the sampling times and in Crisphead lettuce 2/7 (28.57%) of the sampling times. None was detected in the cabbage, broccoli and parsley samples.

Shigella spp., *Campylobacter* spp. and coliform counts were found abundantly throughout the season.

Table 62 The monthly variation in presumptive bacterial counts (in CFU/g) on cabbage collected from farm B, over a one-year period

Produce/ Sampling Date	SH	SAL	LM	EC	TC	CAMP
Cabbage						
Jul-09 - Oct-09	ND	ND	ND	ND	ND	ND
Nov-09	3.93	N	N	N	4.63	4.67
Dec-09	4.63	N	N	N	4.73	4.05
Jan-10	4.02	N	N	N	4.91	4.34
Feb-10 - May-10	ND	ND	ND	ND	ND	ND
Jun-10	3.93	N	N	N	4.68	4.61
Chinese cabbage						
Jul-09	ND	ND	ND	ND	ND	ND
Aug-09	ND	ND	ND	ND	ND	ND
Sep-09	3.56	N	N	N	3.79	3.64
Oct-09 - Jun-10	ND	ND	ND	ND	ND	ND
Red cabbage						
Jul-09	4.92	N	3.53	N	4.06	4.20
Aug-09	3.53	N	N	N	N	3.72
Sep-09	N	N	N	N	4.00	3.71
Oct-09	N	N	N	N	4.28	4.11
Nov-09 - Jun-10	ND	ND	ND	ND	ND	ND
Broccoli						
Jul-09	N	N	N	N	N	N
Aug-09	3.50	N	N	N	5.69	6.37
Sep-09	3.87	N	N	N	3.78	4.50
Oct-09- Feb-10	ND	ND	ND	ND	ND	ND
Mar-10	4.06	N	N	N	3.87	4.61
Apr-10 - Jun-10	ND	ND	ND	ND	ND	ND
Crisphead lettuce						
Jul-09	4.17	N	N	N	N	3.50
Aug-09	4.33	N	N	N	6.11	6.15
Sep-09	ND	ND	ND	ND	ND	ND
Oct-09	4.90	N	N	N	5.37	5.91
Nov-09	ND	ND	ND	ND	ND	ND
Dec-09	N	N	N	N	N	4.90
Jan-10 - Mar-10	ND	ND	ND	ND	ND	ND
Apr-10	N	N	N	N	N	4.81
May-10	N	3.52	N	N	N	4.76
Jun-10	4.09	3.73	3.63	3.54	4.81	5.03
Spinach						
Jul-09 - Jan-10	ND	ND	ND	ND	ND	ND

Produce/ Sampling Date	SH	SAL	LM	EC	TC	CAMP
Feb-10	3.96	3.51	N	N	3.81	4.09
Mar-10	4.24	3.57	N	N	3.92	4.19
Apr-10	4.41	3.51	N	3.50	3.99	4.37
May-10	4.27	3.59	N	N	3.77	4.13
Jun-10	ND	ND	ND	ND	ND	ND
Parsley						
Jul-09 - Sep-09	ND	ND	ND	ND	ND	ND
Oct-09	4.71	N	N	4.64	4.02	4.64
Nov-09	N	N	N	N	4.30	3.83
Dec-09 - Jun-10	ND	ND	ND	ND	ND	ND
Bell pepper						
Jul-09 - Jan-10	ND	ND	ND	ND	ND	ND
Feb-10	3.96	3.51	N	N	3.81	4.09
Mar-10	4.24	3.57	N	N	3.92	4.19
Apr-10	4.41	3.51	N	3.50	3.99	4.37
May-10	4.27	3.59	N	N	3.77	4.13
Jun-10	ND	ND	ND	ND	ND	ND

SH = *Shigella* spp.; SAL = *Salmonella* spp.; LM = *L. monocytogenes*; EC = *E. coli*; TC= Total coliforms; CAMP = *Campylobacter* spp.; ND = Not determined as the fresh produce was not present at the time of sample collection; N = bacterial species was not detected.

Fresh produce from farm C

On Farm C only cabbage was sampled over a three month period (July-September 2009).

Coliform counts of the produce were mostly unacceptable according to the DoH guidelines for ready-to eat fresh produce (Table 63).

E. coli – none detected over the sampling period.

L. monocytogenes – none detected over the sampling period.

Salmonella – none detected over the sampling period.

Campylobacter spp. and Presumptive *Shigella* spp. was found to be abundant in the fresh produce, with the highest concentration being recorded at 6.47 log CFU/g in the cabbage sample during winter (July 2009). No fresh produce samples were available for collection from farm C from March 2010-June 2010, as this farmer specialises in orange farming.

Table 63 The monthly variation in presumptive bacterial counts (CFU/g) on fresh produce collected from farm C, over a one-year period

Produce/ Sampling Date	SH	SAL	LM	EC	TC	CAMP
Cabbage						
Jul-09	6.47	N	N	N	4.81	3.79
Aug-09	N	N	N	N	5.63	6.29
Sep-09	3.95	N	N	N	N	3.51
Oct-09 - Feb-10	ND	ND	ND	ND	ND	ND

SH = *Shigella* spp.; SAL = *Salmonella* spp.; LM = *L. monocytogenes*; EC = *E. coli*; TC= Total coliforms; CAMP = *Campylobacter* spp.; ND = Not determined as the fresh produce was not present at the time of sample collection; N = bacterial species was not detected.

The results of bacterial counts on fresh produce from Farm A, B and C were summarised in Table 64.

Table 64 Range of bacterial load (CFU/g) of the fresh produce over a 12 month sampling period.

Fresh produce	Bacterial *spp.*					
	Coliforms	***E. coli***	***L. monocytogenes***	***Salmonella***	***Shigella spp.***	***C. jejuni***
Broccoli	3.50-4.69	0	0		0-0.43	0-6.37
Cabbage/ Red cabbage & Chinese cabbage	3.64-6.25	0-4.62	0	0-4.15	0-5.00	3.64-6.25
Cauliflower	6.05	0	0	0	5.50	4.97
Crisphead lettuce	0-6.11	0-5.97	0-4.05	0-4.31	0-5.94	3.12-6.15
Jam tomatoes	0-4.40	0	0	0	3.81-5.01	3.75-4.64
Parsley	0-6. 37	0-5.01	0-3.84	0-4.92	0-5.32	3.79-6.41
Spinach	3.77-3.99	0-3.50			0-4.71	0-4.61

Discussion and conclusion

The guideline limit for the incidence of *E. coli* in irrigation water is ≤1000 CFU/100 mℓ for crops not to be eaten raw and allowed to dry (DWAF, 1996). According to these standards the irrigation water sources (river, borehole, dam) on all three farms were acceptable for irrigation of fresh produce to be eaten raw. Interestingly, presumptive *Shigella* spp. were detected in all the water samples from all three farms (A, B and C). Presumptive *Campylobacter* spp. was only detected in water sample C (dam water). The population increased when the irrigation water was not in use, and the dam was allowed to stagnate. This probably allowed for the growth and survival of *Campylobacter* spp., being a microaerophile (Moore *et al*., 2006), since stagnant waters limit the entrance of oxygen. Furthermore, temperature and solute concentration govern the water solubility of oxygen. It has been noted that the water quality

depends partially on land use and how these water resources are managed and protected, as some pathogenic microorganisms may survive longer in water or soil when conditions are optimal than what has been considered to be the norm.

The attachment of pathogenic bacteria to surface areas is important in their survival as they can integrate themselves into biofilms and thus be protected from harsh conditions. It has been reported that seasonal effects may have an impact on the survival of pathogens, with the different seasons of the year having a direct effect on the contaminated state of fresh produce by way of a change in the climatic temperature, rainfall, farming practices (such as fertiliser application) (Hall *et al*., 2002), and changes in the level of ultra-violet irradiation (Griffin *et al*., 1999). Also, the frequency of irrigation is affected by seasonal temperatures, therefore when fresh produce surfaces are in direct contact with irrigation water, an increase in the microbial load as well as a higher threat of contamination can be expected (Stine *et al*., 2005).

The availability of water in the soil can lead to an increase in the microbial population in plant tissues as a result of higher turgor of plants, higher plant transpiration rate and subsequent moisture accumulation on the leaf surface (Fonseca and Rushing 2006). This could explain why the highest concentrations of microbial contamination were obtained in summer, in most of the produce obtained from the different farms. High microbial counts were evident in most of the plants tested, with presumptive *Campylobacter* spp. and *Shigella* spp. being the most abundant microorganisms. However, presumptive *Campylobacter* spp. was not detected in the irrigation water of farm A (river and borehole) and B throughout the study but was evident in the fresh produce and in the soil. The leafy vegetables such as lettuce and spinach had the most microbial contamination compared to broccoli and jam tomatoes. Crops that are grown closer to the ground are much more susceptible to contamination since they can come into direct contact with the contaminant, through splashed soil or manure during irrigation (Hanning *et al*., 2008). Leafy vegetables with large leaf surface areas have also been shown to have a high water holding capacity and are therefore at a greater risk of contamination during irrigation (Ilic *et al*., 2009). Results obtained showed that produce was more heavily contaminated towards maturation.

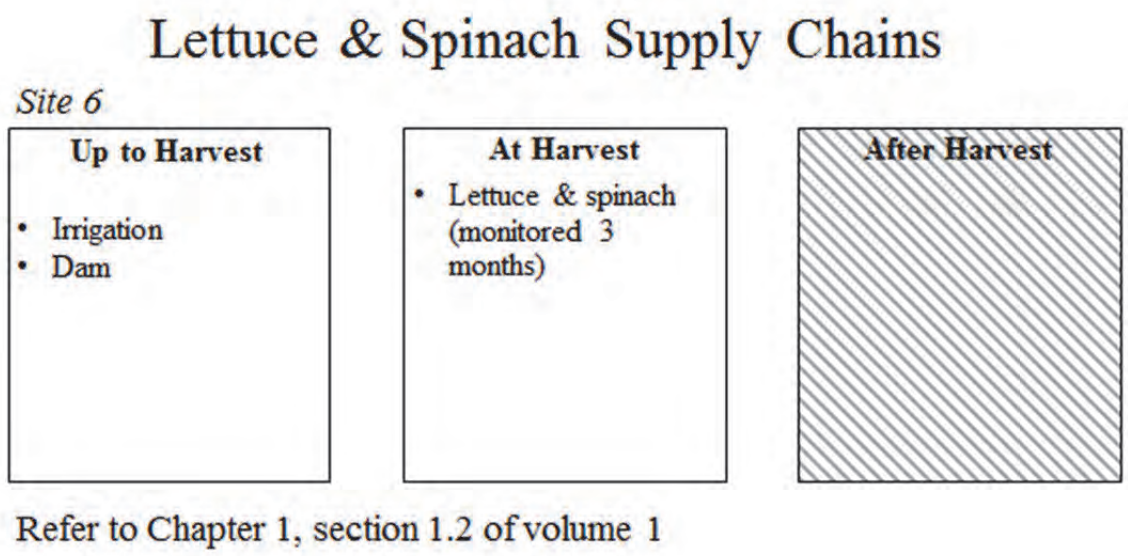
3.2.6 KWAZULU-NATAL PROVINCE – Irrigation water quality (including agrichemical spray water) and the microbiological quality and safety of leafy vegetables (spinach and lettuce) on a farm near Cato Ridge

Specific aim

The motivation of this study was to gain a broad understanding on the microbial ecology of fresh produce and how factors such as irrigation water and pesticides used during cultivation could result in contamination by foodborne pathogenic bacteria resulting in a decrease in food quality and shelf life.

Experimental procedures

Sampling site



Sample collection and processing

Water:

Irrigation water was sampled once a month from a farm in the Cato Ridge area, KwaZulu-Natal for a period of 3 months (August-October, 2013). The size of the farm is 60 hectares and their source of irrigation is dam water. Water was collected from the tap used for irrigation in ethanol-sterilised 5 ℓ plastic bottles.

Sample collection and processing

Lettuce and spinach samples were obtained monthly from a farm in the Cato-Ridge area, KwaZulu-Natal over a period of 3 months (August-October, 2013). The farm is 60 hectares and their source of irrigation is dam water. The crops were irrigated twice a week. The crops were randomly collected using sterile gloves from 3 points in a cultivated field, where one of the fresh produce samples was collected at each point.

Ten grams of each fresh produce sample collected from the field was used for enumeration of microbial load. The leafy fresh produce collected was washed by shaking thoroughly with 90 mℓ of 0.1% (w/v) of sterile peptone water for 30 s, allowed to rest for 30 s and shaken again for another 30 s (Gemmell and Schmidt, 2012).

Microbiological analysis of irrigation water samples

Water, spinach and lettuce samples collected from the farm was enumerated by carrying out 10 fold serial dilutions and spread plating onto selective media (as described in section 3.2.5).

Measuring the physical parameters of water

BOD (biological oxygen demand) and COD (chemical oxygen demand) was determined using HACH HQ40d Portable Meter with HACH (LC101 probe) and a spectroquant NOVO 60 photometer respectively, according to standard methods (Clesceri *et al*., 1998). Total dissolved solids (TSD), turbidity and pH was determined using a HACH HQ40d portable meter with a HACH probe CDC 401, 2100 P HACH turbimeter and Beckman 50 pH meter.

Influence of pesticides in agrichemical spray water on the microbial load found in irrigation water

Pesticide mixed with irrigation water was collected from the farm. This was then used to enumerate microorganisms found in the solution by preparing 10 fold dilutions of the sample at different time intervals (0 h, 6 h, 12 h, 24 h and 48 h) and plating onto selective mediap as described above.

Results

Water:

Aerobic colony counts (ACC) – the ACC plate counts ranged between 2.8 log CFU/ mℓ (August 2013) to 3.6log CFU/mℓ (October 2013) (Fig. 31). The gradual increase in *E. coli* species detected in irrigation water could be attributed to the increase of BOD, COD, TDS and turbidity from August to October (Table 65).

E. coli – the *E. coli* counts gradually increased from 2.25 log CFU/mℓ to 2.5 log CFU/mℓ.

Presumptive *L. monocytogenes* and *Salmonella* spp. were present in water sampled from August 2013-October 2013.

Presumptive *Shigella* spp. were only isolated during September and October.

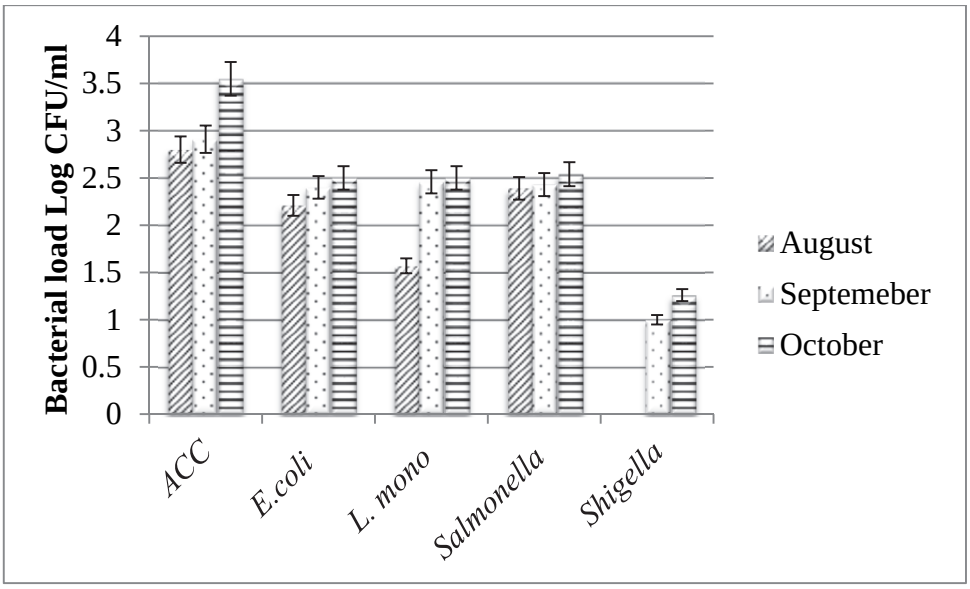


Figure 31 Presumptive bacterial species detected in irrigation water for 3 months

Table 65 Physical parameters of irrigation water

Physical parameters	August	September	October
Temperature °C	20	23	26
pH	6.9	7.1	7.23
Salinity ‰	0.23	0.25	0.28
Turbidity UNT	228	238	286
TDS mg/ℓ	16.8	17. 5	18. 4
BOD mg/ℓ	6.5	6.6	7.02
COD mg/ℓ	250	253	262

Lettuce and spinach:

Aerobic colony counts (ACC) – the ACC counts ranged between 4.11 and 4.5 log CFU/g over the three month sampling period (Fig. 32).
E. coli – The *E. coli* counts obtained for spinach ranged from 3.3-3.7 log CFU/g and for lettuce ranged from 3.8-4.6 log during the sampling period (Fig. 33).

Presumptive *L. monocytogenes*, *Salmonella* and *Shigella* were present on lettuce and spinach and cabbage from August 2013-October 2013 (Fig. 34-36).

Combined microbiological profile of irrigation water and fresh produce:

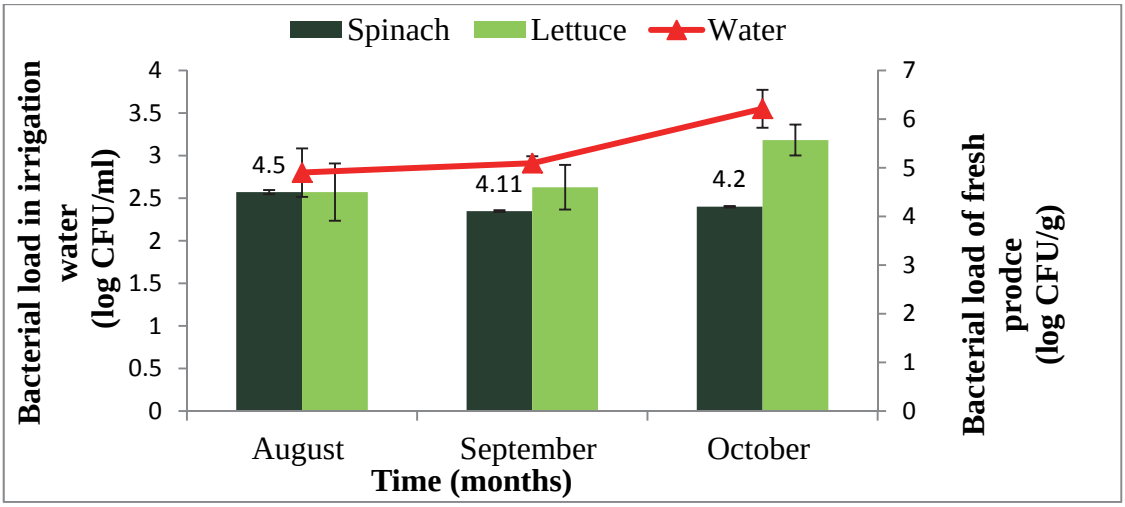


Figure 32: Average ACC detected in irrigation water and fresh produce during the sampling period

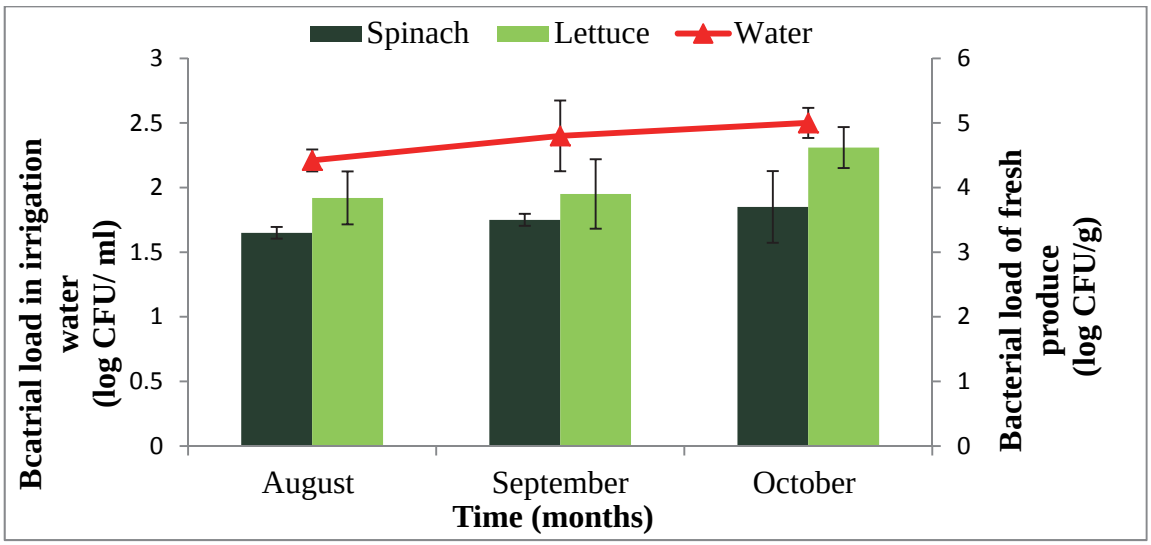


Figure 33: Average *E. coli* counts detected in irrigation water and fresh produce

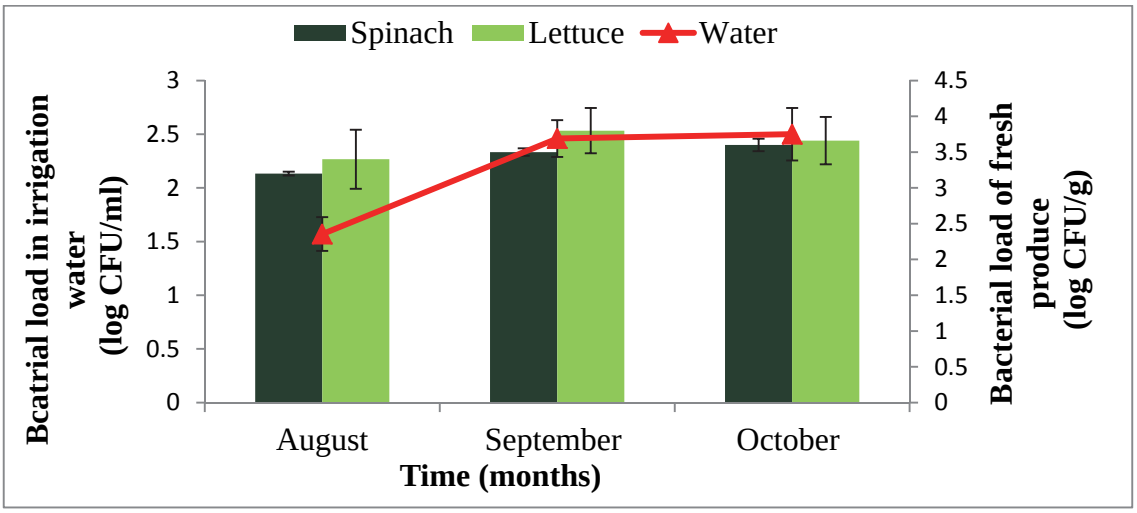


Figure 34: Average presumptive *L. monocytogenes* detected in irrigation water and fresh produce during the sampling intervals

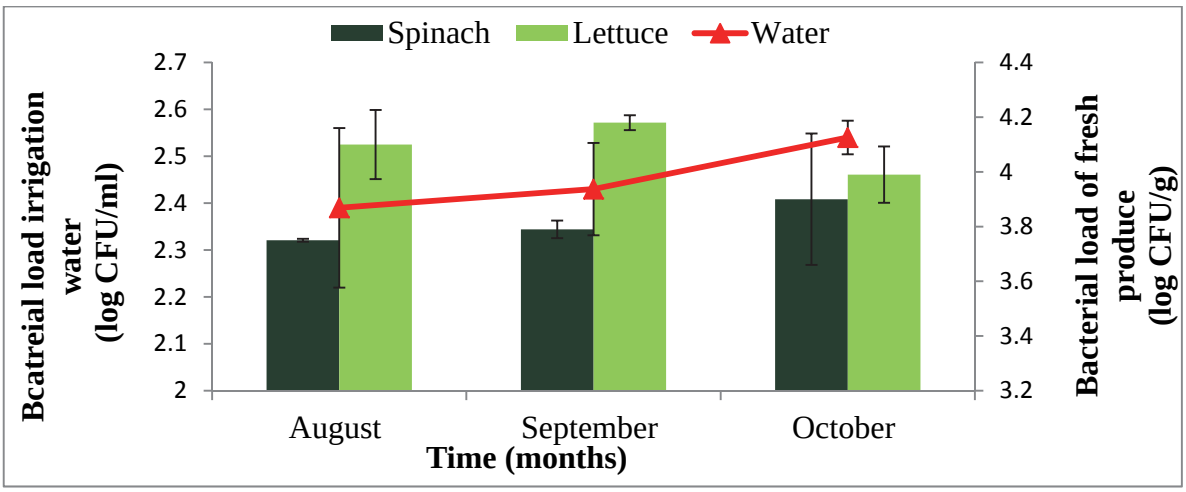


Figure 35: Average presumptive *Salmonella* counts detected in irrigation water and fresh produce during the sampling interval

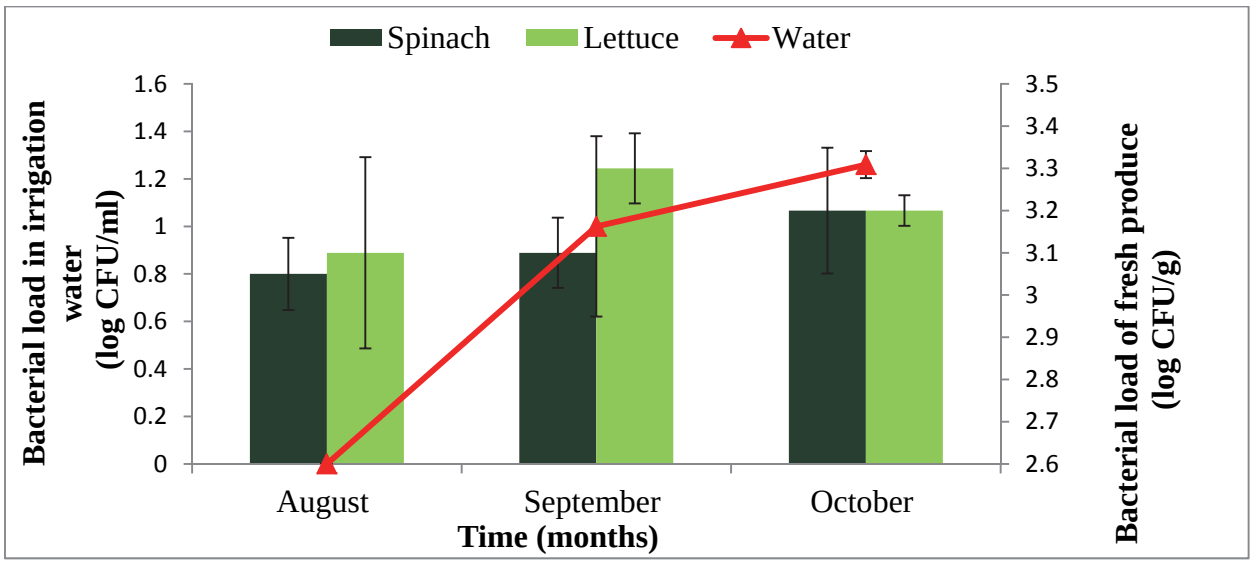


Figure 36: Average presumptive *Shigella* counts detected in irrigation water and fresh produce

Influence of pesticides in agrichemical spray water on the microbial load found in irrigation water

Agrichemical sprays used on the farm showed the ability to inhibit growth of some of the microorganisms found in irrigation water. As indicated in Figure 37 below *E. coli* and ACC were the only species detected in the pesticide solution. *E. coli* increased from 2.5 log CFU/mℓ at time 0 to 3.4 log CFU/mℓ after 6 hours of incubation. The *E. coli* of water compared to the pesticide solution is low, indicating that pesticides do have the potential to promote growth of some microorganisms. ACC counts of water is very low, when compared to the pesticide solution. This can be attributed to the fact that these contain a wide range of microorganisms some of which have the ability to utilize the active ingredient found in the pesticide as a nutrient source. After 12 hours the *E. coli* levels decreased. However, the ACC counts started decreasing at 24 hours.

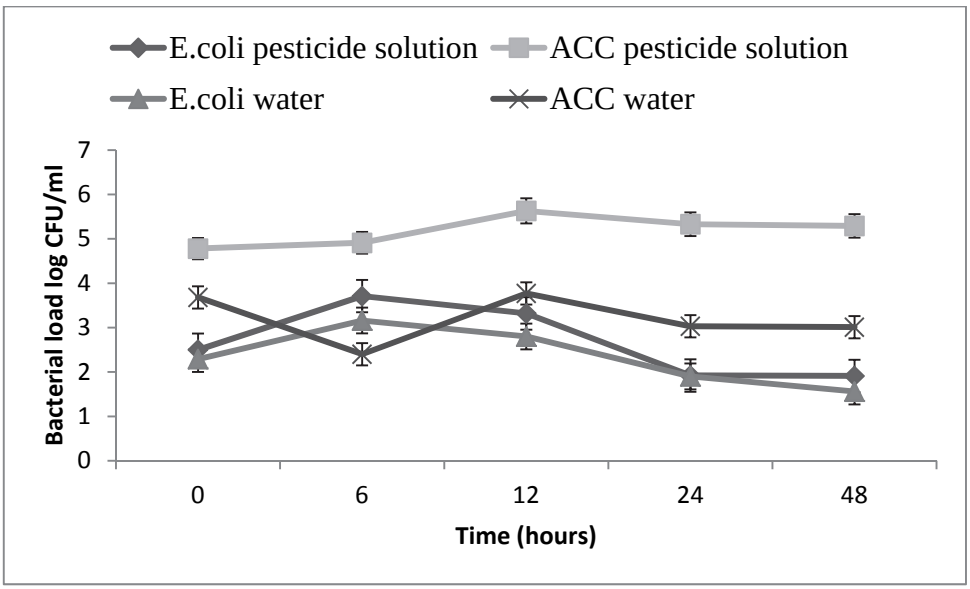


Figure 37: Presumptive microbial species detected on alpha-trin pesticide mixed with irrigation water

Discussion and conclusion

Temperature plays a pivotal role in the growth of bacteria, as bacteria have different optimum temperatures for growth. The low counts observed for most of the bacteria in August might have been due to the low temperature of water and the atmospheric temperature as this was still the late stage of winter. As the temperature increased from August to October there was a gradual increase in *E. coli* numbers in the irrigation water and on the lettuce. The presence of human pathogenic bacteria in irrigation water and on fresh produce is of concern as they have been linked with a number of foodborne illnesses in the past.

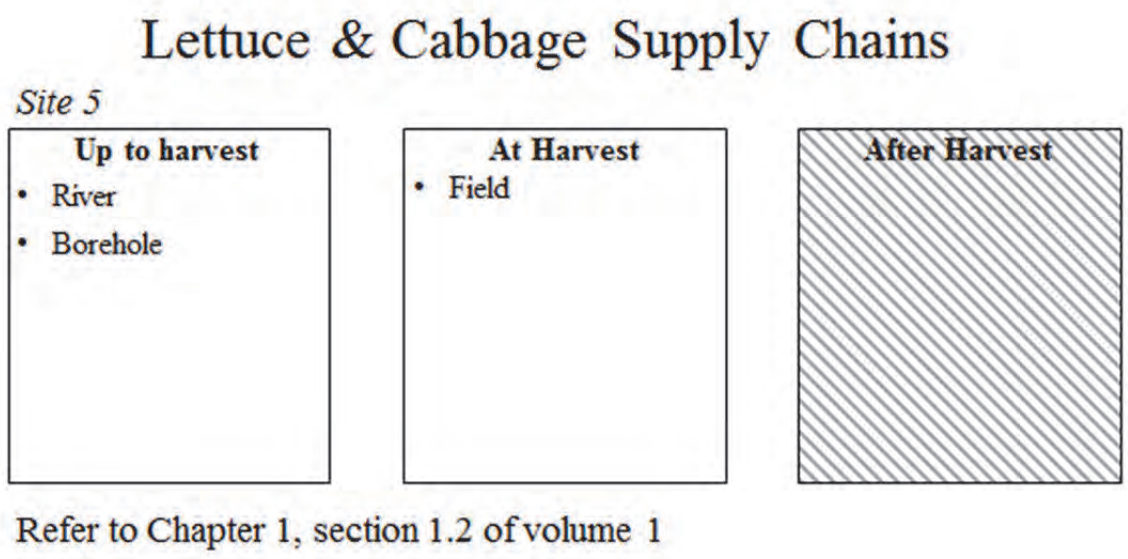
The increase in the total ACC and *E. coli* counts in the irrigation water mixed with pesticides is similar to results obtained by Peter *et al*. (2005). They showed the ability of pesticides to promote growth of some microorganisms when mixed with irrigation water.

3.2.7 KWAZULU-NATAL PROVINCE – Irrigation water quality (including agrichemical spray water) and the microbiological quality and safety of fresh produce (cabbage and lettuce) on two farms near Camperdown

Specific aim: To determine the microbiological quality of irrigation water, fresh produce and agricultural spray water and to show relationships among all possible pairs of physico-chemical parameters [BOD (biological oxygen demand) ,COD (chemical oxygen demand), total dissolved solids (TSD), turbidity and pH] and the bacterial counts found on the crops.

Experimental procedures
Sampling sites

Farms near Camperdown KwaZulu-Natal.



Sample collection

Irrigation water and agricultural spray water samples were collected from two farms in KwaZulu-Natal for a period of three months (July-September 2014). Irrigation water and pesticide solutions were collected using sterile 2 L plastic bottles pre-washed with 70% ethanol and rinsed with the sample before collection. After collection, all the samples were stored on ice during transportation to the laboratory where they were stored at 4°C and processed for analysis within 24 hours (Gemmell and Schmidt, 2012).

Lettuce (*Lactuca sativa* L.) and cabbage (*Brassica oleracea* L.) were collected randomly from three different points in the cultivated field on both farms using sterile gloves and packed into sterile re-sealable plastic bags (Tirawat *et al*., 2013). Potable water samples and home grown lettuce were donated for analysis and comparison to the samples from the two farms. These samples were collected using the same methods as described for the farm samples. After collection, all the samples were stored on ice during transportation to the laboratory where they were stored at 4°C and processed for analysis within 24 hours (Gemmell and Schmidt, 2012).

Microbiological analysis of irrigation water samples

Sample water collected from the farm was enumerated by carrying out 10 fold serial dilutions and spread plating onto selective media as described in section 3.2.5.

Measuring the physical parameters of water

BOD (biological oxygen demand), COD (chemical oxygen demand), total dissolved solids (TSD), turbidity and pH were determined as described in section 3.2.5.

Impact of pesticides on the microbial load found in irrigation water

Pesticides mixed with irrigation water were collected from both farms. One hundred microliters of the pesticide solution were placed in sterile flasks and incubated at 37°C, 24 h and shaking at 150 rpm. Samples were then taken at 0, 12 and 24 h intervals and bacteria enumerated as previously mentioned. Microbial counts were expressed as CFU/ml. Table 66 illustrates the pesticide mixtures and their compositions based on the sampling period. At each sampling period the combination of pesticides was different.

Table 66 Different pesticides used and their active ingredients.

Farm	Pesticides used (600 L)	Type	Active ingredient
A1	Metfos 585 SL	Insecticide	Methamidophos
	Aqua 5 Right	Buffer	Buffering and wetting systems; Alcohol ethoxylate
	Aphox	Insecticide	Pirimicarb
B1	Biohumate	Fertilizer	Humic acids
	Dimethoate	Insecticide	Dimethoate
	Urea HB	Fertilizer	Nitrogen
	Aqua 5 Right	Buffer	Buffering and wetting systems; Alcohol ethoxylate
A2	Aqua 5 Right	Buffer	Buffering and wetting systems; Alcohol ethoxylate
	Aphox	Insecticide	Pirimicarb
	Nu-Film P	Non-toxic sticker – spreader	Poly-1-p-menthene
	Metfos 585 SL	Insecticide	Methamidophos
	Liquid boron	Fertilizer	Boron
B2	Allice 20 SP	Insecticide	Acetamiprid (neonicotinoid)
	M45 dithane	Fungicide	Mancozeb
	Biohumate	Fertilizer	Humic acids
	Urea HB	Fertilizer	Nitrogen
	Aqua 5 Right	Buffer	Buffering and wetting systems; Alcohol ethoxylate

Farm	Pesticides used (600 L)	Type	Active ingredient
A3	Prev – AM	Fungicide / insecticide /miticide	Borax; Orange oil
	Aphox	Insecticide	Pirimicarb
	Belt	Insecticide	Flubendiamide (phthalic acid diamide)
	Bolldex	Biological bollworm control	Helicoverpa armigera nucleopoly-hedrovirus (HearNPV)
	Nu-Film	Non-toxic sticker – spreader	Poly-1-p-menthene
	Agromectin	Insecticide	Abamectin
	Aqua 5 Right	Buffer	Buffering and wetting systems; Alcohol ethoxylate
B3	Steward 150	Insecticide	Indox acarb – oxadiazine
	Allice 20 SP	Insecticide	Acetamiprid (neonicotinoid)
	Aqua 5 Right	Buffer	Buffering and wetting systems; Alcohol ethoxylate
	Odeon 720 SC	Fungicide	Chlorothalonil
	Urea HB	Fertilizer	Nitrogen
	Afrikelp LG-1	Plant growth stimulant	Ecklonia maxima (liquid seaweed concentrate)
	Cyperin 200 EC	Insecticide	Cypermethrin (pyrethroid)
A4	Prev – AM	Fungicide / insecticide /miticide	Borax; Orange oil
	Aphox	Insecticide	Pirimicarb
	Belt	Insecticide	Flubendiamide (phthalic acid diamide)
	Bolldex	Biological bollworm control	Helicoverpa armigera nucleopoly-hedrovirus (HearNPV)
	Aqua 5 Right	Buffer	Buffering and wetting systems; Alcohol ethoxylate

Farm	Pesticides used (600 L)	Type	Active ingredient
B4	Decis Forte	Insecticide	Deltam ethrin (pyrethroid)
	Urea HB	Fertilizer	Nitrogen
	Aqua 5 Right	Buffer	Buffering and wetting systems; Alcohol ethoxylate
	Agromectin	Insecticide	Abamectin
	Voema Calmag and TE	Fertilizer	Microelements
	Diclorvos 1000 EC	Insecticide	Dichlorvos
	M45 dithane	Fungicide	Mancozeb

A and B = Farm A and Farm B respectively 1-4 = Sampling periods

Confirmation of presumptive pathogen identities

DNA isolation

Genomic DNA isolation was done using the boiling method according to Ahmed *et al*., (2014).

PCR amplification and confirmation of isolates

PCR amplification of DNA from *L. monocytogenes* was performed according to Jeyaletchumi *et al*. (2010). For the amplification of the *mdh* gene in *E. coli*, the protocol used by Roohollah and Mahjar (2013) was used. For the amplification of the *InvA* gene in *Salmonella* spp., the protocol by Gassama-Sow *et al*. (2006) was used. Details of primers used for PCR analysis were summarised in Table 67.

Table 67 The description of the primers, sequences and target genes.

Organism	Target gene	Primers	Primer sequence (5’ ⟹ 3’)	Product size (bp)
E. coli	*mdh*	*mdh*-F	CGTTCTGTTCAAATGGCCTCAGG	392
		mdh-R	ACTGAAAGGCAAACAGCCAAG	
L. monocytogenes	*hlyA*	*hlyA*-F	CAAGTCCTAAGACGCCAATC	1412
		hlyA-R	ATAAAGTGTAGTGCCCCAGA	
Salmonella spp.	*InvA*	*InvA*-F	TGCCTACAAGCATGAAATGG	450
		InvA-R	AAACTGGACCACGGTTGACAA	

Statistical analysis

Data of the log averages and associated standard deviations (SD) from each set of three replicates were calculated for each sample time using Excel 2010 (Microsoft). The data was statistically analysed using mean and standard deviations. P-values associated with T-test and One-way ANOVA was taken as an indicator of significant differences among the data using GraphPad Prism software (version 5.00). In all statistical analyses, confidence level was held at 95% and $P<0.05$ (at 5% level of significance) was considered as significant, Pearson product-moment correlation coefficient using SPSS (SPSS, Inc., Illinois 21.0) was used to show relationships among all possible pairs of physico-chemical parameters and the bacterial counts found on the crops.

RESULTS

Water:

Total Heterotrophic bacteria (THB) – the THB plate counts in water from both farms were similar and ranged between 2.91 log CFU/ml - 3.49 log CFU/ml. (Fig. 37).

E. coli – the *E. coli* counts ranged between 0.52 log CFU/ml and 1.4 log CFU/ml (Fig. 38).

Presumptive *L. monocytogenes* and *Salmonella* were present in the irrigation water samples (Fig. 39).

The identities of presumptive *E. coli*, *Salmonella* and *L. monocytogenes* were confirmed using PCR analysis and pathogen specific primers as described in the Experimental procedures section.

Lettuce and cabbage

Total Heterotrophic bacteria (THB) – HTB counts ranged from 3.88-7.21 and 3.08-6.74 log CFU/g for lettuce and cabbage, respectively (Fig.38). Lettuce generally had a higher microbial load than cabbage.

E. coli counts on cabbage ranged between 0.52-1.12 log CFU/g and on lettuce between zero and 3.2 log CFU/g (Fig. 39).

Presumptive *L. monocytogenes* and *Salmonella* were present on some of the lettuce and cabbage samples (Fig. 39).

Profile of irrigation water, potable water and fresh produce

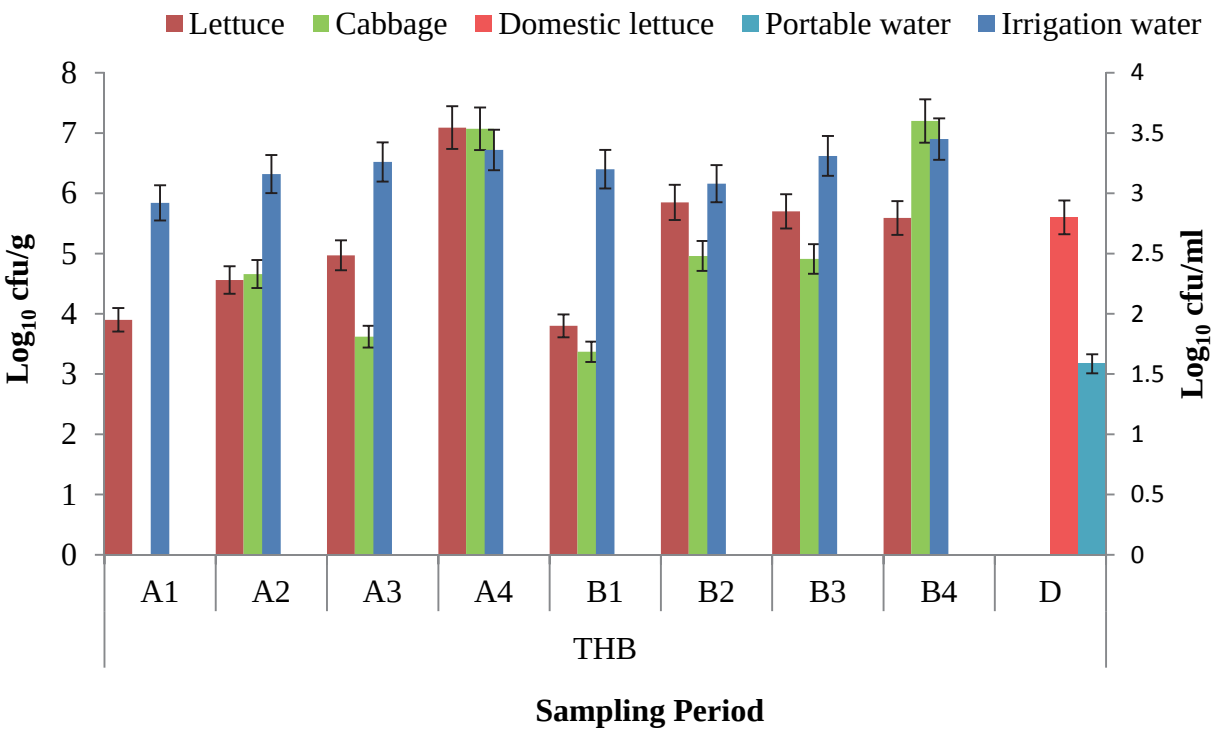


Figure 38: Microbial load of total heterotrophic bacteria in irrigation water and fresh produce.

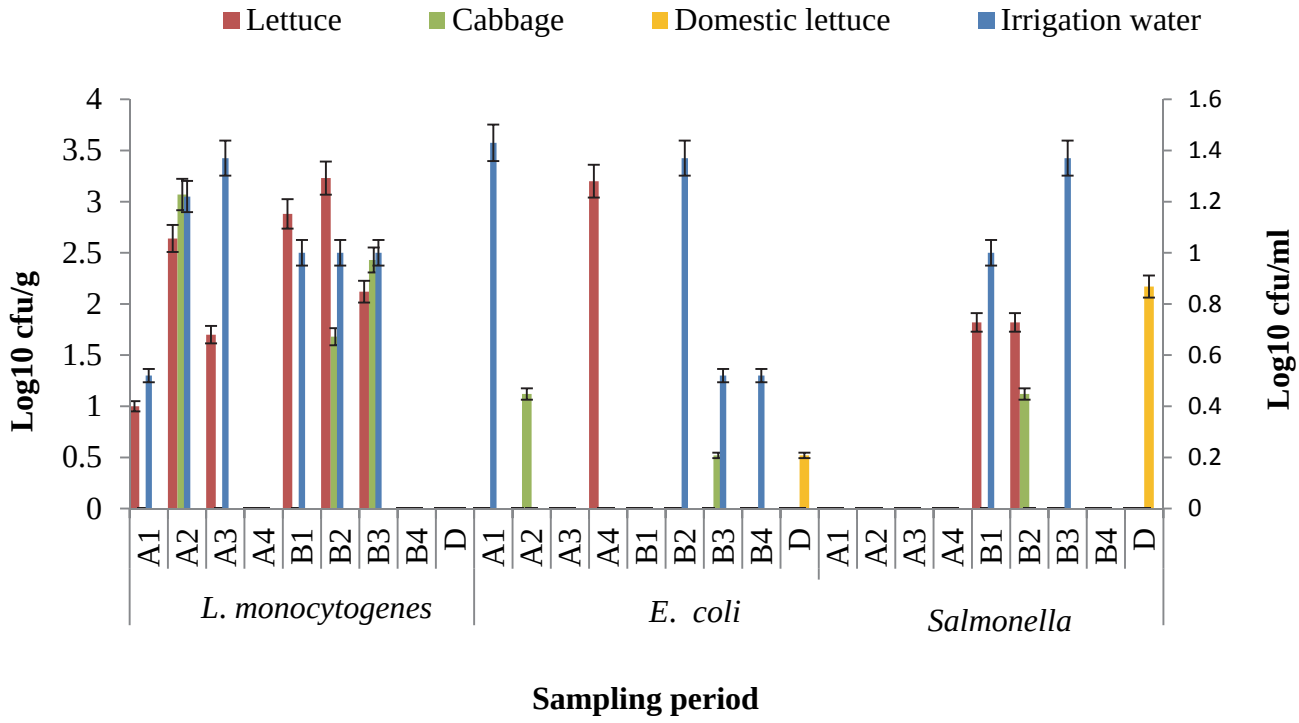


Figure 39: Microbial load of selected bacteria in irrigation water and fresh produce.

Physico-chemical properties of water samples

The data for the physic-chemical parameters of water obtained for both farms and the potable water was summarised in Table 68. The water temperature from all three sites was similar and ranged from 15-20°C during the research period. The pH of the irrigation water from farm A ranged between 7.02-7.98 while it ranged between 7.0.4-7.47 on farm B. TDS of the water samples differed significantly ($p < 0.01$) during the four sampling periods for both farms. The turbidity of the water samples from both farms differed significantly ($p < 0.05$). Farm B had a higher mean turbidity of 2.94 NTU compared to farm A which had a mean turbidity of 2.74 NTU. The COD and BOD values of the water samples were not significantly different ($p > 0.05$). Farm A had a higher mean COD of 155.7 mg/l compared to farm B which had a lower mean COD of 80.54 mg/l. The physico-chemical parameter data for the potable water was within the standards for drinking water.

Table 68 Physico-chemical parameters of irrigation (A & B) and potable water (D) samples

Sample	Temp (°C)	pH	Turb (NTU)	TDS (mg/l)	COD (mg/l)	BOD_5 (mg/l)
A1	17	7.12	2.48 ± 0.04	150.93 ± 0.40	302 ± 4.58	0.79 ± 0.01
A2	16	7.02	2.60 ± 0.08	158.70 ± 0.61	86 ± 3.00	0.96 ± 0.01
A3	17	7.58	2.83 ± 0.03	155.67 ± 0.15	107.33 ± 5.69	0.62 ± 0.01
A4	20	7.98	3.00 ± 0.21	161.43 ± 0.06	127.33 ± 19.09	3.01 ± 0.01
B1	16	7.04	1.85 ± 0.04	193.33 ± 0.15	50.5 ± 2.12	0.67 ± 0.02
B2	15	7.43	2.99 ± 0.01	198.97 ± 0.15	219.33 ± 2.31	0.99 ± 0.01
B3	16	7.27	3.75 ± 0.05	192.50 ± 0.20	12.00 ± 3.00	4.99 ± 0.02
B4	20	7.47	3.15 ± 0.05	193.3 ± 0.10	40.33 ± 1.15	1.17 ± 0.01
D	19	7.35	0.5 ± 0.11	72.07 ± 0.15	8 ± 1.00	6.75 ± 0.01

Turb: Turbidity, Temp: Temperature, TDS: Total dissolved solids, COD: Chemical oxygen demand, BOD: Biochemical oxygen demand

The data in Table 69 shows that there was a positive and significant correlation ($p \leq 0.05$) between pH and turbidity (r= 0.952), between pH and temperature (r= 0.899), and between temperature and turbidity (r= 0.782). Positive correlation was also noted between COD and *E. coli* counts in the water samples (r= 0.985). There was a positive and highly significant ($p \leq 0.01$) correlation between the COD and THB counts (r= 0.995), and between the THB and *E. coli* counts (r= 0.998) in the water samples. TDS influenced the growth of *E. coli* (r= 0.650), *L. monocytogenes* (r= 0.381) and THB (r= 0.616). This was illustrated by the positive correlation between these factors. There was a positive correlation between various

parameters, including BOD and temp, pH and turbidity (r= 0.926, 0.764 and 0.725, respectively).

Table 69 Correlation matrix of physical parameters of irrigation water for farm A.

	Temp	pH	Turb	TDS	COD	BOD	THB	LM	EC
Temp	1								
pH	.899	1							
Turb	.782	.952*	1						
TDS	-.760	-.703	-.786	1					
COD	-.030	-.301	-.579	.555	1				
BOD	.926	.764	.725	-.921	-.200	1			
THB	-.127	-.396	-.658	.616	.995**	-.281	1		
LM	-.670	-.284	-.073	.381	-.551	-.681	-.493	1	
EC	-.192	-.459	-.709	.650	.985*	-.331	.998**	-.454	1

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

SAL (*Salmonella* spp.) is constant. THB: total heterotrophic bacteria, LM: *L. monocytogenes,* EC: *E. coli*

From the data in Table 70 it can be seen that there is a highly significant ($p \leq 0.01$) correlation between COD and TDS on farm B (r= 0.998). Significant correlation ($p \leq 0.05$) was observed for *E. coli* with TDS and COD, as indicated by r= 0.971 and r= 0.956, respectively. A positive correlation was observed between *Salmonella* spp. and pH (r= 0.983), and turbidity (r= 0.591). There was a negative correlation between *L. monocytogenes* and all the physico-chemical parameters with the exception of temperature (r= 0.980), with which there was a significant positive correlation ($p \leq 0.05$).

Table 70 Correlation matrix of physical parameters of irrigation water for farm B.

	Temp	pH	Turb	TDS	COD	BOD	THB	EC	SAL	LM
Temp	1									
pH	-.717	1								
Turb	-.075	.466	1							
TDS	-.079	-.428	.170	1						
COD	-.093	-.390	.224	.998**	1					
BOD	-.337	.057	-.857	-.435	-.474	1				
THB	-.293	.840	.807	-.382	-.329	-.423	1			
EC	-.066	-.543	-.071	.971*	.956*	-.231	-.581	1		
SAL	-.594	.983*	.591	-.442	-.398	-.095	.925	-.589	1	
LM	.980*	-.596	-.084	-.273	-.285	-.257	-.187	-.260	-.471	1

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).
SAL: *Salmonella* spp., THB: total heterotrophic bacteria, LM: *L. monocytogenes*, EC: *E. coli*

Pesticide analysis

The data in figure 40 shows an increase in the microbial load from 0-24 hours for A1, A3, A4 and B1, while A2, B2, B3 and B4 showed a decrease. The pesticide mixture used at each sampling interval was different. In B4 no initial counts were observed at 0 hours, however there were very high counts at 12 hours followed by a sharp decrease in the microbial at 24 hours of incubation. In A2 there was total inhibition of the microbes at 24 hours, with B2 having no growth at 12 hours. The bacterial counts ranged from 1.99-5.42, 2-7.25, and 0.52-8.07 at 0, 12 and 24 hours respectively. Data recorded for the 24 hour incubation were not significant ($p > 0.05$).

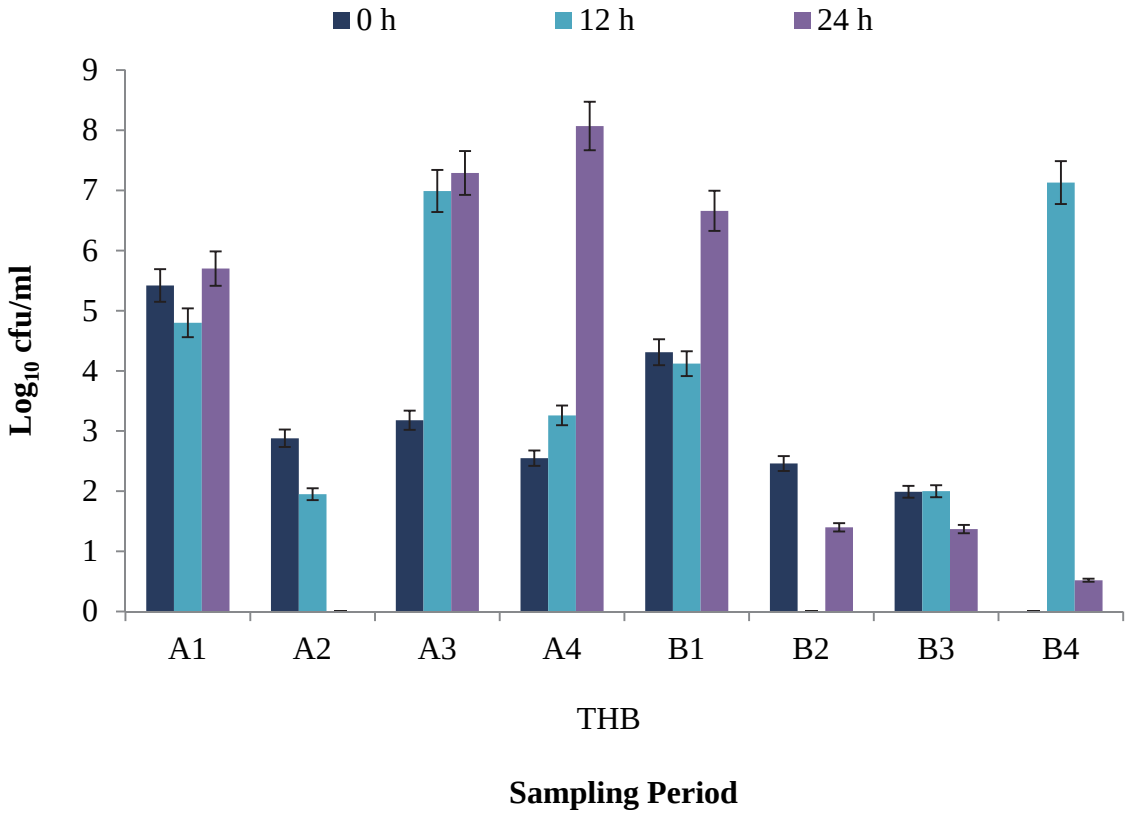


Figure 40: Microbial load of THB in irrigation water mixed with pesticides at different time periods.

The data in Table 71 shows that there was initially inhibition of the selected microbes at 0 h, however at 12 h *E. coli* and *Salmonella* were detected in A3 and B4 sampling intervals. Growth of all three microbes was observed at 24 hours for A1 and A3 sampling intervals. A common trend was observed with pesticides used in A1 and A3 sampling intervals.

Table 71 Microbial load of selected bacteria in irrigation water mixed with pesticides at different time periods in $\log_{10}$ CFU/ml.

Organism	Sampling Period	Pesticide solution sampling time (h)		
		0	**12**	**24**
L. monocytogenes	A1	0	0	0.52
	A2	0	0	0
	A3	0	0	0.52
	A4	0	0	0
	B1	0	0	0
	B2	0	0	0
	B3	0	0	0
	B4	0	0	0
E. coli	A1	0	0	2.54
	A2	0	0	0
	A3	0	1.12	3.26
	A4	0	0	0
	B1	0	0	0
	B2	0	0	0
	B3	0	0	0
	B4	0	0	0
***Salmonella* spp.**	A1	0	0	2.34
	A2	0	0	0
	A3	0	2	2.70
	A4	0	0	0
	B1	0	0	0
	B2	0	0	0
	B3	0	0	0
	B4	0	1.12	0

A and B = farm A and farm B. 1-4 = sampling times.

Discussion and conclusion

The pH of all the irrigation water samples fell within the recommended range of the Food and Agriculture Organization of the United Nations (FAO) of pH 6.5-pH 8.4 (FAO, 1996). Water temperature was in the range of 15-20°C, including the potable water sample. This was expected as the sampling was done during the months of July-September when temperatures are not very high. The pH affects functioning of the cell. Since the pH was close to neutral values, this created an ideal environment for the growth of most of the organisms, including enteric pathogens. The mean turbidity levels in the irrigation water exceeded the international turbidity of 0-1 NTU standard for water (DWAF, 1996). The turbidity of the potable drinking water was within the range of 0-1 NTU. The high turbidity levels of the irrigation water could be the result of soil erosion and runoff. There was a positive correlation between the BOD, temperature and pH, and the turbidity levels on farm A. The values for TDS recorded on both farms were within the recommended range of 1000 mg/l for TDS in drinking water and 40 mg/ml for irrigation water as specified by WHO (WHO, 1996). There was a positive correlation between the TDS and COD levels in the irrigation water from both farms. *E. coli* counts appeared to be influenced by TDS and COD on farm B. Microbial growth could have been supported by the presence of organic matter in the irrigation water.

The BOD levels were lower than the COD levels which is expected since BOD only measures organic material compared to COD which measures both inorganic and organic matter in the water samples. There was a negative correlation between COD and BOD levels on both farms. COD and TDS values were the only two parameters of water that seemed to have influenced the survival of the THB in the irrigation water on farm A. TDS seemed to have also influenced the survival of *E. coli*, *Salmonella* spp. and *L. monocytogenes* in the irrigation water sources on both farms. There was a significant positive correlation between the COD levels and the THB and *E. coli* counts in the water samples. South Africa does not have any guidelines for COD and BOD. However, the COD values recorded exceeded those recommended by WHO of 10 mg/l. This is an indication that the water contains organic pollutants which could help promote the growth of certain microbes in the water. There was a positive correlation between the THB and *E. coli* in the water on farm A while there was a negative correlation between the two in water from farm B. THB counts were also positively correlated wth the *Salmonella* counts. This could be as a result of mutualistic relationships between *Salmonella* and some of the other bacteria in the sample. These other bacteria could be breaking down nutrients for *Salmonella* or it could be using the by-products of these other organisms that are present in the environment.

South Africa does not have standards for the amount of aerobic counts allowed in irrigation water; however the guidelines for domestic water specify a heterotrophic plate count of ≤ 100 CFU/ml (DWAF, 1996). The difference in mean counts was found to be significant ($p \leq 0.05$) for irrigation water and vegetables from farm A, and highly significant ($p < 0.01$) for irrigation water and vegetables from farm B. There was no significance ($p \geq 0.05$) in the mean values of potable water and the lettuce sample. The heterotrophic bacterial counts are in

general used as an indication of the microbial quality of water. THB counts are not a representation of the total number of bacteria present in a sample.

Contamination of water sources by other bacterial pathogens, namely, *L. moncytogenes* and *Salmonella* showed that the irrigation water samples are of poor microbial quality. The presence of the indicator *E. coli* in the water samples evaluated indicates possible contamination of the water sources by faecal matter. The international standard for faecal coliforms allowed is 1000 CFU/100 ml in irrigation water, and this was exceeded in the majority of the samples. The microbial load on lettuce was generally higher compared to cabbage samples. Slight differences in the micro-architectural structure of the vegetable may also contribute to differences in pathogen attachment and survival. Supporting this statement, higher numbers of *Salmonella* spp. attached to Romaine lettuce compared to cabbage surfaces (Monaghan and Hutchison, 2012). Lettuce irrigated with non-potable water has been shown to have significantly higher rates of total coliforms and presumptive *Salmonella* contamination than lettuce irrigated with drinking water (Strawn *et al*., 2013). Chai *et al*., (2011) showed that *Salmonella* spp. present on the surface of plants can survive for extended periods and might overcome the immune response of plants and actively enter through the stomata. This was demonstrated for lettuce. No presumptive *Shigella* spp. was detected in all of the samples analysed. *Shigella* is not ubiquitous in the environment and contamination of fresh produce by this species has been mostly linked to improper sanitation and human handling (Nygren *et al*., 2013).

The use of pesticides to control weeds, insects, and other pests has resulted in a range of benefits, including increased food production. South Africa has one of the largest markets for pesticides in Sub-Saharan Africa (Quinn *et al*., 2012). The data for the pesticide analysis showed that the active ingredients of the pesticides could act as inhibitors or promoters of microbial growth. These findings correlate with previous studies which showed that pesticide solutions provide a suitable environment for the survival and growth of pathogenic microbes, such as *L. monocytogenes*, *E. coli*, *Salmonella* and *Shigella* spp., whereas others are inhibitory to these microorganisms (Campos *et al*., 2013). The common trend was that the microbes' growth was favoured mainly by the pesticides with organophosphates and carbamates as the active ingredients. Organophosphate pesticides contain diazinon and chlorpyrifos. Other active ingredients were abamectin (16-membered macrocyclic lactone derivative), phtalic acid diamide and poly-1-p-methene (insect growth regulator). The effect of combining more than one pesticide in the water might be different than the effects of each individual pesticide alone. The frequency of the application of pesticides and the degree of adsorption has been shown to affect microbial degradation of pesticides and utilising it as a source of nutrients.

3.2.8 LIMPOPO PROVINCE – Potential link between irrigation water quality and the microbiological quality and safety of onions and tomatoes from a commercial farm near Polokwane

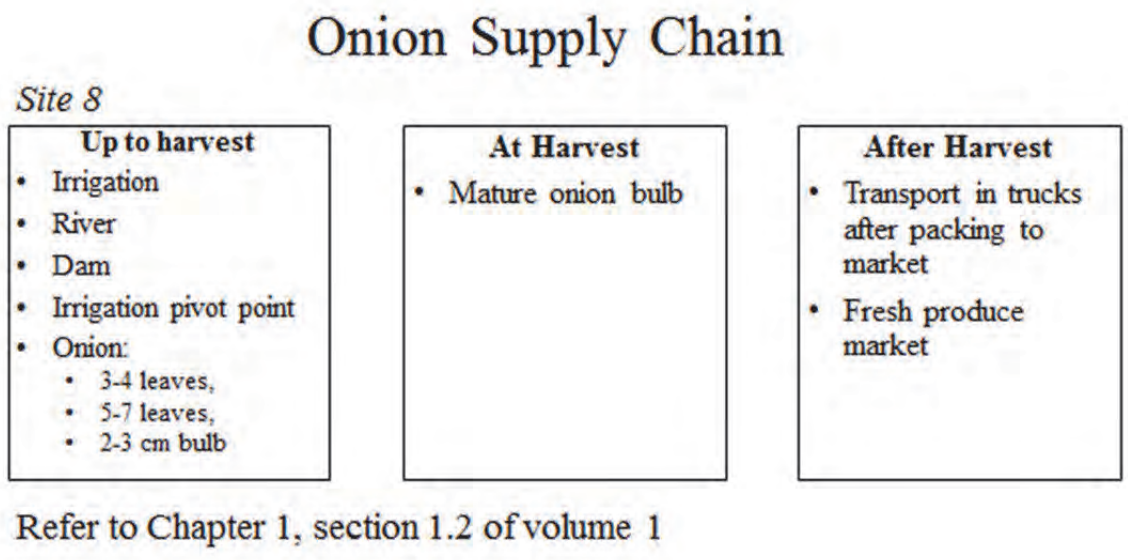
Specific aim

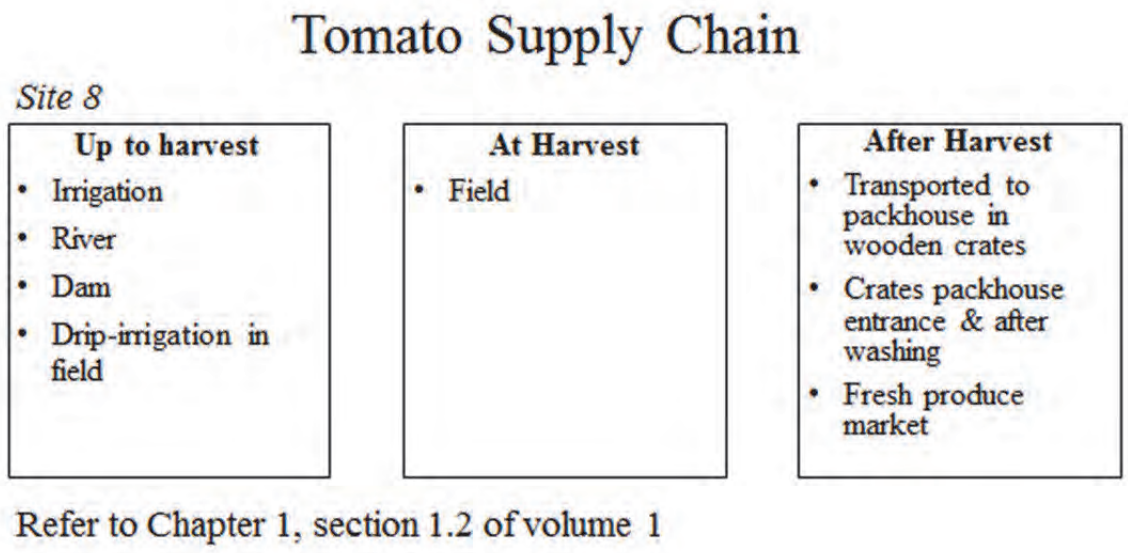
The aim of this study was to determine the microbiological quality, prevalence of human pathogenic bacteria. The study also aimed to characterise the *Escherichia coli* isolates from irrigation water (river, associated water storage dam and water from overhead irrigation pivot point) as well as onions, in order to establish a possible link between the water quality and the potential transfer of human pathogens from the irrigation source to the fresh produce.

Experimental procedures

Experimental site

A commercial onion/tomato production farm situated near Polokwane in the Limpopo Province. Irrigation water was pumped from the Sand River to holding dams and used for overhead irrigation of the onions. The tomatoes are grown in open fields. Irrigation water is pumped from the Sand River into a catchment dam and pumped through black PVC pipes into the field where tomatoes are irrigated using drip-irrigation. Tomatoes are harvested, transported to the packhouse, washed with Sporekill-treated water and hand-packed into boxes. The tomatoes are transported to the Tshwane Fresh Produce Market in non-refrigerated trucks, where it is sold to the retail and hospitality industries the next day.





Water sampling and processing

From January to August 2012 water samples were collected from a selected river, water storage dam and an overhead irrigation pivot point on a commercial fresh produce farm in the Limpopo Province, South Africa. The river forms part of the Limpopo River basin in the same Province. Samples, (3 x 1 L) per sampling site, were collected in ethanol (70%) sterilized, air dried plastic bottles from each of the sites. Samples were transported in a cooler box and stored at 4 °C until analysis (within 24 h). All analyses were performed in triplicate. Each of the one litre water samples was filtered through cellulose nitrate filters (0.45 µm pore size) (Sartorius, Goettingen, Germany) for further microbiological analysis.

Onion sampling and processing

Onion replicate samples (comprised of three sub-samples per replicate) were systematically sampled from four rows in the onion field at different growth stages, i.e. 3-4 leaves, 5-7 leaves and 2-3 cm bulb size (before harvest), at harvest and after harvest (from the fresh produce market). The onions were sampled at the different growth stages to determine at which stage/s potential human pathogenic bacteria (*E. coli* (including O157:H7), *Salmonella* spp. and *Listeria monocytogenes*), present in the irrigation water, were detected in onions. The harvested whole onions were placed in labelled bags and then transported to the laboratory as described for the water samples. The outer dry leaves of the mature onions were removed and the onions chopped up using a sterile knife and cutting board. Three twenty-five grams sub samples of each replicate were weighed into a sterile polyethylene bag containing 225 mℓ of 0.1% peptone buffered water (BPW) (Merck, Johannesburg, South Africa) and macerated for 5 min at 230 rev min-1 in a Seward Stomacher 400 Circulator (Seward Ltd., London, UK).

Tomato sampling and processing

Samples were collected at harvest, in the field, from plants at three different locations in the same block. At the packhouse tomatoes were sampled from wooden crates at the entrance and after washing. Specific boxes were marked on the day of packing. Tomatoes from the marked

boxes were sampled at the fresh produce market the following day. All samples were taken in triplicate and five tomatoes represented a single sample.
Each tomato sample was weighed, one litre Ringer's solution supplemented with Tween-80 added and sonicated in an ultrasonic bath for 5 min. The fruit was removed and the solution vacuum filtered through a 45 µℓ membrane. Each tomato sample was washed in an ultrasonic bath in one litre Ringer's solution supplemented with Tween-80. The fruit was removed and the solution vacuum filtered through a 0.45 µM filter membrane.
The washed tomatoes were then halved and a half of each tomato blended to a purée with an electric blender. Twenty-five g purée was transferred to stomacher bags containing 225 mℓ PBW. The bags were stomached for 2 minutes.

Microbiological analysis
Each 0.45 µM filter membrane was cut into small pieces, added to 9 mℓ TSB medium and serially diluted. Bacteria, fungi and yeasts on tomato surfaces were numerated as described in Section 2.3.1 (microbiological analysis).
The stomached tomato (25 g) samples were enriched by incubation at 37°C for 24 hours to facilitate detection of human pathogenic bacteria.

Confirmation of microorganism identities using MALDI-TOF MS
Coliform colonies were isolated from 3M Petrifilm media (3M), streaked onto EMB agar to obtain single colonies and incubated for 24h at 37°C. The identities of the 24 hour isolates were determined using Matrix-assisted laser desorption ionization time-of flight mass spectrometry (MALDI-TOF). Purified bacterial cultures isolated from the selective media were transferred in duplicate directly to the MALDI-TOF steel polished target plate (Bruker, Bremen, Germany), and overlaid with the α-cyano-4-hydroxycinnamic acid matrix (Bruker). The target plate was subsequently analysed using Bruker MicroFlex LT MALDI-TOF in conjunction with Bruker Biotyper Automation Software and library. The MALDI-TOF was calibrated prior to use with the bacterial standard supplied by Bruker. Duplicate score values (SV) were recorded; SV were used to determine the accuracy of identification. A SV of between 1.999 and 1.700 was used to identify the genus name of the organism, and a value of above 2.0 was used to determine the genus and probable species of an organism. Above 2.3 is highly probable species identification.

Molecular methods
DNA was extracted from single colonies of presumptive *E. coli*, *Salmonella* and *Listeria* isolates using the Quick-gDNA miniprep kit (Zymo Research, California, USA). The universal primers F-27 and R-1492 were used to amplify the 16s rDNA genes of the bacterial isolates (Lane *et al*., 1985). The PCR products were sequenced and a search of nucleotide databases conducted to confirm the microorganism identities.

Antibiotic resistance of *E. coli* isolates
Confirmed *E. coli* isolates from water and fresh produce were subjected to antibiotic susceptibility testing using the Kirby Bauer disk diffusion technique (Bauer *et al*., 1966). Confirmed *E. coli* isolates were cultured in Brain Heart Infusion Broth at 37°C for 24 hour and hundred microlitres of the bacterial suspension were plated onto Mueller-Hinton agar

plates (Merck). The antibiotic agents and concentrations used are summarised in Table 72 (Mast Diagnostics, UK, supplied by Davies Diagnostics, Midrand, SA). Antibiotic resistance or susceptibility of *E. coli* isolates were scored according to criteria developed by the National Committee on Clinical Laboratory Standards (CLSI 2007).

Table 72 Summary of antibiotic agents and concentrations used in this study

Class	Antibiotic	Abbreviation	Concentration
Penicillins	Augmentin (Amoxicillin / Clavulanic Acid)	AUG30C	20 µg / 10 µg
	Ampicillin	AP10C	10 µg
	Aztreonam	ATM30C	30 µg
	Cefepime	CPM30C	30 µg
	Cefotaxime	CTX30C	30 µg
	Cefquinome	CEQ30C	30 µg
	Ceftazidime	CAZ30C	30 µg
Cephems	Ceftriaxone	CRO30C	30 µg
Phenicols	Chloramphenicol	C30C	30 µg
Quinolones	Enrofloxacin	ENF5C	5 µg
Quinolones	Nalidixic Acid	NA30C	30 µg
	Florphenicol	FFC30C	30 µg
Tetracyclines	Tetracycline	T30C	30 µg
Folate Pathway Inhibitors	Cotrimoxazole (Trimethoprim Sulfamethoxazole)	TS25C	1.25 µg / 23.75 µg

Virulence genes in *E. coli* isolates from irrigation water and onions

The same *E. coli* isolates used for determining antibiotic resistance were tested for the presence of virulence genes [shigatoxin 1 (*stx* 1), shigatoxin 2 (*stx* 2) and intimin (*eae*)] and the house-keeping *mdh* gene (internal PCR reaction control) as described by Omar and Barnard (2010).

Enterobacterial Repetitive Intergenic Concencus (Eric) PCR fingerprinting of *E. coli* isolates

Forty five presumptive *E. coli* colonies isolated from river, dam and irrigation water samples as well as onions were analysed respectively. The DNA of presumptive *E. coli* isolates colonies was extracted and the DNA concentrations were determined using a Qubit 2.0 Fluorometer (Lifescience Technology, Johannesburg, South Africa). DNA concentrations were adjusted to 80 µg/µℓ for ERIC PCR analysis. Two reference strains were included in the analysis of the ERIC PCR, *E. coli* O157:H7 (ATCC 35150) and *E. coli* JM109.

A T100™ Thermal Cycler (Bio-Rad, Johannesburg, South Africa) were used for amplifications to perform the ERIC PCR analysis. Reactions were performed in 50µℓ volumes that included 160ng of template DNA, 50*p*mol of the ERIC primer. ERIC forward and reverse primer sequences were 5'- ATGTAAGCTCCTGGGGATTCAC -3' and 5'-

AAGTAAGTGACTGGGGTGAGCG -3' respectively. The PCR cycling conditions involved an initial denaturation step of 95°C for 4 min, 30 cycles of 94°C for 30 s, 40°C for 1 min, 72°C for 8 min and a final elongation step at 72°C for 15 min. The PCR products were held at 4°C until removal of the product.

Statistical analysis

Analysis of variance and correlations coefficients were calculated to determine associations between the measured traits using XLSTAT version 2014. To group the populations based on antibiotic phenotype similarities, a cluster analysis was conducted on the Euclidean distance matrix with the unweighted Pair Group Method based on Arithmetic Averages (UPGMA) using XLSTAT 2014.

The Eric PCR DNA fingerprints of *E. coli* isolates were compared and analysed with the GelCompar v. 6.5 (Applied Maths, NV). Percentage similarities of digitised bands were calculated using the Pearson correlation coefficient and the unweighted pair group arithmetic mean (UPGMA) and complete linkage algorithms were used to derive a dendrogram. Relationships between the DNA fingerprint profiles of each of the isolates were expressed as dendrograms.

Results

Microbiological analysis of water used to irrigate onions

Aerobic colony counts (ACC) – The ACC counts varied between 2.5 log CFU/mℓ to 6.5 log CFU/mℓ (Sand River); from 3.5 log to 6 log CFU/mℓ (dam) and from 3.1 log CFU/mℓ to 5.5 log CFU/mℓ (onion irrigation pivot point) and 3.1 to 3.8 log CFU/mℓ (micro irrigation pipe in the tomato field) (Fig. 41). ACC reflects the total microbial content including spoilage organisms (Sela and Fallik, 2009). DWAF guidelines state that water for safe domestic use should have heterotrophic bacterial counts of less than 2 log CFU/mℓ

Coliforms and E. coli. Coliform and *E. coli* counts were higher during summer than during winter (Fig. 41).

River water: *E. coli* counts on the farm exceeded 1000 CFU/100 mℓ during the summer months. However, the *E. coli* counts during the winter months were close to or at acceptable levels according to the guidelines of ≤ 1-1000 CFU/ 100 mℓ for irrigation water (DWAF, 1996) (Fig. 41).

Dam water: *E. coli* counts exceeded the irrigation water guidelines only once during week 7, (February 2012). This water source is used to irrigate onions and tomatoes (Fig. 41).

Irrigation pivot point water sampled when the onions were irrigated during March (week 13), May (week 20) and June (week 25) *E. coli* counts complied with guidelines of ≤ 1-1000 *E. coli* CFU/100 mℓ (Fig. 41).

Drip irrigation pipe water: the tomatoes were produced and irrigated in summer and the *E. coli* levels for one of the sampling intervals exceeded 1000 CFU/100 mℓ.

Fungi – Fungal counts were low ranging from a minimum of 0.1 log CFU/mℓ(pivot point water) to a maximum of 2.6 log CFU/mℓ (dam water).

Yeast – Yeast counts ranged from a minimum of 0.75 log CFU/mℓ(dam) to a maximum of 4.5 log CFU/mℓ (river).

E. coli O157:H7 – None isolated.
Salmonella spp. – Isolated from 22% of the water samples tested included *Salmonella enterica* subsp. enterica serovar Typhimurium.
Listeria spp. – None isolated.
Noro viruses (NoV GI, NoVG1) and Hepatitis A virus (HAV)
River: all three viruses were detected in the river water for 5/6 (January, March, May, June, August) sampling intervals.
Dam: all three viruses were detected in the river water for 3/6 sampling intervals
Irrigation pivot point: Nov GI was detected in 1/3 (May 2012) sampling intervals.
Nov GII and HAV was detected for 3/3 (March, May and June 2012) sampling intervals.

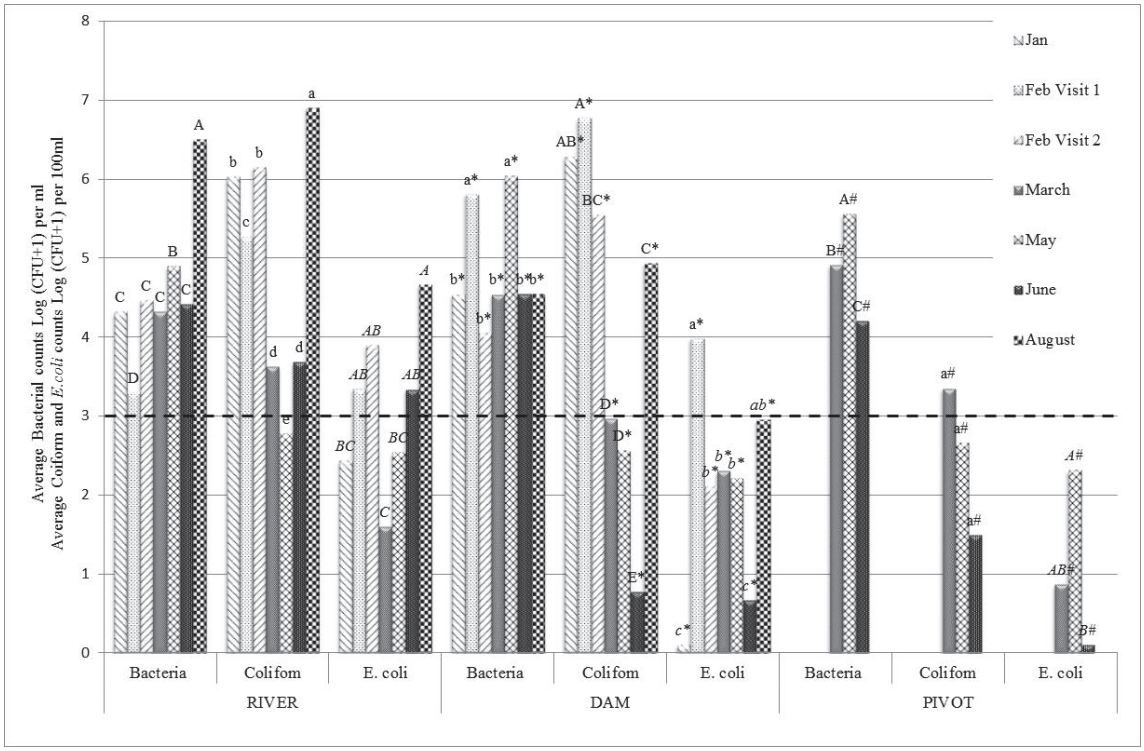


Figure 41: Aerobic bacteria, coliform and *Escherichia coli* counts for river, dam and irrigation pivot point water. The broken line indicates the South African Department of Water Affairs (1996) guideline limit for irrigation water of ≤ 1-1000 *E. coli* CFU/100 mℓ.

Determination of the physical parameters in the irrigation water
The pH, conductivity, ammonium, nitrate, nitrite, orthophosphate and COD of all the water samples were determined for the field trips on 31/01/2013 and 13/08/2013 and are summarised in Table 73. The electrical conductivity (EC) of the water samples ranged from 70.9 m Siemens/m to 115.2 mS/m which can be regarded as good and tolerable respectively according to the DWAF irrigation water guidelines (DWAF, 1996). Nitrogen occurs predominantly as nitrate in irrigation water. The ammonium form is usually a result of contamination with waste water. The highest level of ammonium (25.5 mg/ℓ) was indeed recorded for the water sampled from the dam 3 outflow just below the Polokwane waterworks (sample 5). The waterworks often overflow after rain leading to contamination of the river

water downstream, which is used for irrigation purposes on the farm. The pH of all the water samples tested were within the recommended pH range for irrigation water in South Africa, i.e. from pH 6.5 to pH 9.5 (DWAF, 1996). However, this range also promotes growth of enteric pathogens.

Table 73 Summary of physical properties of the irrigation water sources

Sample number			River upstream	River below sewage works	River on farm	Dam 1 on farm	Dam 2 On farm	Dam 3 on farm	23
Physical and aggregate properties									
Date		**Unit/s**							
2012/01/31	pH @ 25°C	pH units	7.5	7.4	7.7	7.8	8.5	8.1	7.2
2012/08/13			7.5	7.4	7.7	8.7	8.4	8.6	7.5
2012/01/31	Conductivity @ 25°C	mS/m	78.6	108.8	83.3	113.6	115.2	70.9	89.6
2012/08/13			124.2	138.7	132.6	115.0	126.0	119.5	114.6
Inorganic non-metallic constituents									
2012/01/31	Ammonium as NH4-N	mg/ℓ	4.8	25.5	10.6	<0.03	<0.03	7	9.3
2012/08/13			18.5	36.8	26.8	0.08	0.37	21.1	18.5
2012/01/31	Nitrate as NO3-N	mg/ℓ	2.7	<1.4	1.8	8.1	10	2.1	5.6
2012/08/13			3.8	<1.4	1.5	2.2	10.8	<1.4	13.3
2012/01/31	Nitrite as NO2-N	mg/ ℓ	0.45	0.01	0.57	0.07	0.09	0.64	0.98
2012/08/13			0.27	<0.01	0.09	0.04	0.15	0.19	0.59
Phosphorus									
2012/01/31	Orthophosphate as PO4-P	mg/ℓ	2.23	4.92	2.91	0.56	0.06	2.52	4.3
2012/08/13			5.79	5.01	4.63	0.62	0.12	4.42	6.18
Aggregate organic constituents									
2012/01/31	COD as O2	mg/ ℓ	42.4	70.7	27.4	<18.1	30.6	41	54.7
2012/08/13			49.5	141	104	<18.1	38	75	97.5

Microbiological analysis of onions

Aerobic colony counts (ACC) – The maximum ACC count on the onion surfaces was 6.9 CFU/g (3-4 leaf stage) and 2 log CFU/g at the fresh produce market (Fig. 42). The onion microbial load gradually declined during the onion production cycle up to marketing, where it was comparable to microbial specification guidelines for fresh fruit and vegetables in South Africa.

Coliforms and E. coli – The total aerobic bacteria and coliform counts decreased significantly from the 3-4 leaf stage to the 2-3 cm bulb stage and the counts for the market onions were not statistically different to the values measured at the 2-3 cm bulb stage (Fig. 42). No *E. coli* was counted for any of the onion samples using coliform/*E. coli* count plates. In contrast, *E. coli* was isolated from 10% of the onion samples, but only after selective enrichment of the onion samples indicating its presence at levels lower than the standard detectable limit for plate counts.

Fungi/ Yeasts – The yeast and fungi counts were very low ranging from 0.77 log CFU/g (lowest) to 1.82 log CFU/g (highest) at the early growth stages, where after the yeast/fungi counts were zero (results not shown).
E. coli O157:H7 – None of the human pathogenic bacteria tested for were isolated from onions at any of the growth stages throughout production and marketing.
Salmonella spp. – None isolated.
Listeria spp. – None isolated

Dominant microorganisms on onion surfaces included *E. cloacae, Pantoea ananatis, Pseudomonas chlororaphis, P. koreensis, Fusarium oxysporum, Penicillium* spp., *Aspergillus niger*, *Microsphaeropsis arundis* and *Alternaria alternata.*

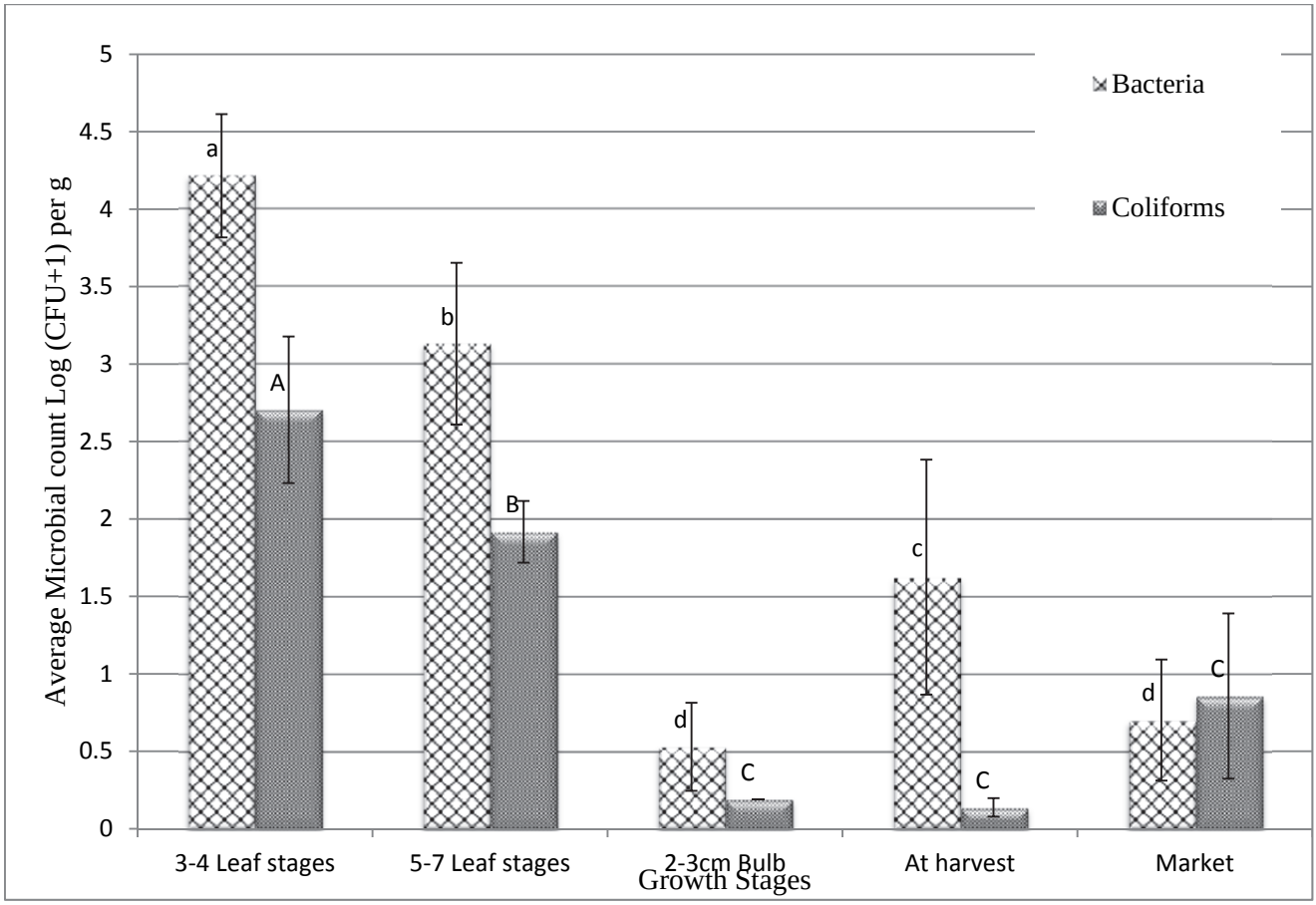


Figure 42: Total aerobic bacteria and coliform counts measured for onions sampled at the different growth stages. *The E. coli* counts of the onions were zero for all the sampling times.

Microbiological analysis of tomatoes

Aerobic colony counts (ACC) – The maximum ACC count was 3.8 log CFU/g for tomatoes after washing in the packhouse and the minimum count 1.8 log CFU/g for tomatoes sampled at the fresh produce market (Fig. 43).

Fungi and yeast counts – fungal and yeast numbers were low, i.e. < or = to 1 log CFU/g = 10 CFU/g (Fig. 43).

E. coli O157H7 and *S.* Typhimurium – None of the human pathogenic bacteria tested for were isolated from tomatoes.

Microorganisms isolated from tomatoes included *Citrobacter freundii, Klebsiella pneumonia, Acinetobacter* genomo species 3, *Serratia marcescens, Enterobacter cloacae, E. aerogenes, E. ludwigii, E. asburiae, Pseudomonas fulva, P. putida, P. stutzeri,* and *P. mendocina.*

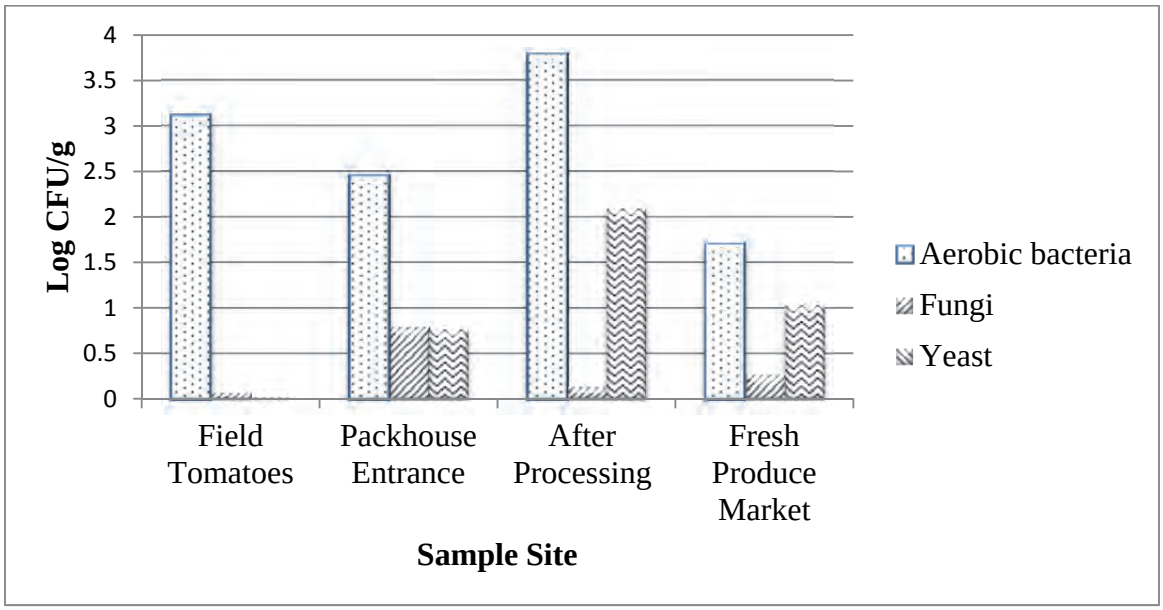


Figure 43 Graph showing the total number of aerobic bacteria, fungi and yeast on the tomatoes.

***Escherichia coli* (including O157:H7), *Salmonella* and *Listeria monocytogenes* prevalence in water, onion and tomato samples**

Selective media used for identification of presumptive *E. coli*, *Salmonella* and *Listeria monocytogenes* after enrichment in appropriate media were not always efficient, since many false positive results were obtained. Presumptive *E. coli* colonies isolated from EMB agar after selective enrichment were identified as either *E. coli*, *Enterococcus hirae*, *Enterobacter cloacae*, *Enterobacter absuriae* and *C. freundii* using MALDI-TOF analysis and 16s ribosomal DNA amplification, sequencing and blast analysis. Results obtained using 16s ribosomal DNA PCR analysis of the colonies (results not shown) showed that 97% of the environmental isolate identities determined using MALDI-TOF analysis were correct. *E. coli* was isolated from 78.4% water samples (n=51) and 8.3% (n=60) onion samples. Typical colonies of *E. coli* O157:H7 were not detected for any of the water and onion samples after streaking onto Agar Chrom ID TM O157H7 (BioMerieux, Marcyl-l' Etoile, France) or PCR

analysis using the *uidA* gene primers targeting the mutated *uidA* gene of *E. coli* O157:H7 as described by Cebula *et al*. (1995).

As was observed for *E. coli* identification the chromogenic media (XLD and SBM) used for identifying presumptive *Salmonella* isolates were not always efficient. MALDI-TOF analysis identifies *Salmonella* only at the genus and not at the species level. Twenty-two percent water samples (n=51) samples harboured *Salmonella* and none were detected in onion samples using MALDI-TOF analysis. These results were confirmed using 16s ribosomal PCR analysis.

All presumptive positive *Listeria monocytogenes* colonies isolated from enriched water and onion samples by streaking onto the Listeria chromogenic media were identified as either *Bacillus* spp., *Enterobacter* spp. or *Staphylococcus* spp. using MALDI-TOF analysis.

Antibiotic resistance of environmental *E. coli* isolates from irrigation water and onions

Forty-five confirmed *E. coli* isolates as well as *E. coli* JM109 and *E. coli* O157:H7 (ATCC 35150) were tested for antibiotic resistance. *E. coli* isolates (n=23) from the river were resistant to 7/14 antibiotics tested (Table74). These included ampicillin, azetronam, chloramphenicol, cotrimazole, enroflaxin, florphenicol, nalidixic acid and tetracycline. *E. coli* isolates (n=14) from water storage dams were resistant to 4/14 antibiotics tested and included ampicillin, florphenicol and tetracycline as well as augmentin resistance which was not observed for any of the other irrigation water and onion *E. coli* isolates. *E. coli* isolates (n=3) from the irrigation pivot point were resistant to 6/14 antibiotics tested (Table 74). These included ampicillin, chloramphenicol, cotrimazole, enroflaxin, nalidixic acid and tetracycline. Although only five *E. coli* strains were isolated from the onions after selective enrichment, they were resistant to the same antibiotics as the river *E. coli* isolates (Table 74).

Table 74 Summary of results obtained for antimicrobial resistance of environmental *E. coli* isolates

		No. (%) of resistant isolates from:			
Antimicrobial(s)	**Amt(s)/disc (µg)**	**River source samples (n=23)**	**Storage Dam samples (n=14)**	**Irrigation pivot samples (n=3)**	**Onion samples (n=5)**
AP 10C	10 µg	9 (39.1)	4 (28.6)	2 (66.7)	2 (40.0)
AUG 30C	20 µg / 10 µg	0 (0)	2 (14.3)	0 (0)	0 (0)
ATM 30C	30 µg	2 (8.7)	0 (0)	0 (0)	2 (40.0)
CPM 30C	30 µg	0 (0)	0 (0)	0 (0)	0 (0)
CTX 30C	30 µg	0 (0)	0 (0)	0 (0)	0 (0)
CEQ 30C	30 µg	0 (0)	0 (0)	0 (0)	0 (0)
CAZ 30C	30 µg	0 (0)	0 (0)	0 (0)	0 (0)
CRO 30C	30 µg	0 (0)	0 (0)	0 (0)	0 (0)
C 30C	30 µg	3 (13.0)	0 (0)	1 (33.3)	1 (20.0)
TS 25C	1.25 µg / 23.75 µg	9 (39.1)	0 (0)	3 (100)	1 (20.0)
ENF 5C	5 µg	1 (4.3)	0 (0)	1 (33.3)	1 (20.0)
FFC 30C	30 µg	3 (13.0)	1 (7.1)	0 (0)	0 (0)
NA 30C	30 µg	2 (8.7)	0 (0)	1 (33.3)	1 (20.0)
T 30C	30 µg	10 (43.5)	5 (35.7)	3 (100)	1 (20.0)

Of the 45 *E. coli* isolates, 7 (15.5%) were resistant to one antibiotic and 19 (42.2%) were resistant to multiple antibiotics (Table 75). One *E. coli* isolate from the overhead irrigation pivot point was resistant to six antimicrobial agents. Except for being sensitive to cotrimoxazole, the antibiotic resistance profile of one of the onion *E. coli* isolates was the same as the profile of the isolate from the overhead irrigation pivot point.

E. coli from the water storage dam showed close phenotypic relatedness based on resistance to antibiotics since most of the dam samples (11/14 isolates) clustered together (Fig. 44). Seven of the river *E. coli* isolates (R5, R19, R29, R36, R38, R20 and R24) and one onion isolate (O30) clustered with the isolates from the dam (Fig. 44).

Table 75 Antimicrobial resistance patterns of *Escherichia coli* isolates from river, storage dams, overhead irrigation pivot point water and onions from a commercial fresh produce farm in Limpopo

No. of antimicrobials to which isolates were resistant	No. (%) of isolates	Most frequent pattern (no. of isolates)	Source (no. of isolates)
0	19 (42.2)		
1	7 (15.5)	ATM 30C (2)	Onion (2)
2	6 (13.3)	AP 10C – T 30C (4)	River (1) Dam (3)
3	5 (11.1)	AP 10C – TS 25C – T 30C (3)	River (2) Irrigation pivot point (1)
4	4 (8.8)	AP 10C – TS 25C – FFC 30C – T 30C (2)	
5	3 (6.6)	AP 10C – ATM 30C – C 30C – TS 25C – T 30C (1) AP 10C – C 30C – ENF 5C – NA 30C – T 30C (1) AP 10C – TS 25C – ENF 5C – NA 30C – T 30C (1)	River River Onion
6	1 (2.2)	AP 10C – C 30C – TS 25C – ENF 5C – NA 30C – T 30C (1)	Irrigation pivot point
All isolates	45 (100)		

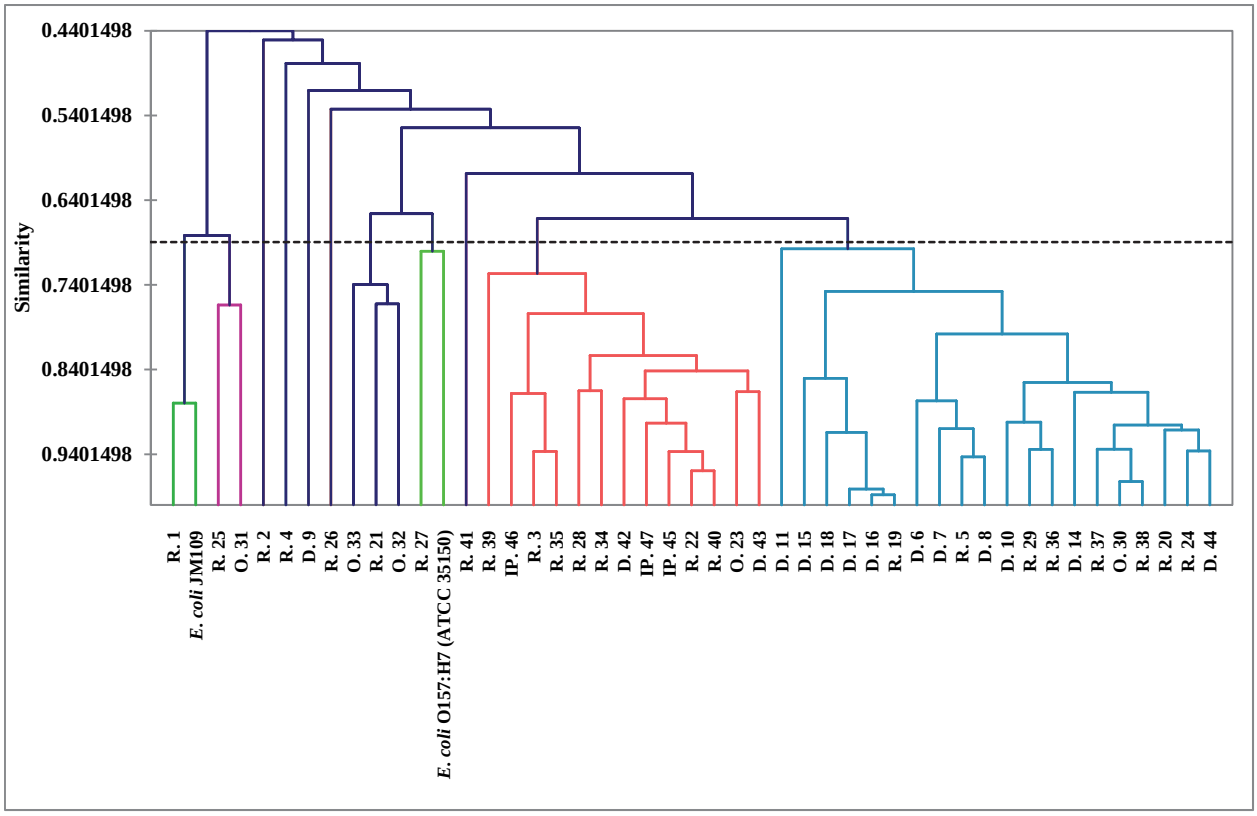


Figure 44: Dendrogram showing cluster formation based on antibiotic resistance profiles for *E. coli* isolated from irrigation water sources (river, dam and irrigation pivot point) and onions.

Virulence gene analysis of environmental *Escherichia coli* isolates from irrigation water and onions

The presence of virulence genes were determined for the same *E. coli* isolates screened for antibiotic resistance. In total 11% of *E. coli* isolates were positive for at least one virulence gene. One *E. coli* river isolate (2.2%) harboured the *eae* gene, three isolates from river, dam and onions respectively (6.6%) harboured the *stx1* gene and 1 river isolate (2.2%) harboured the *stx2* gene.

Enterobacterial Repetitive Intergenic Concencus (Eric) PCR fingerprinting of *E. coli* isolates from irrigation water and onions

A dendrogram showing the genetic relatedness of *E. coli* isolates from irrigation Cluster analysis of Eric-PCR DNA fingerprints generated ten distinct cluster (A-J) (Fig. 45). Several river and dam water samples clustered together in cluster A at similarity values ranging from 64% to 98%. Three river water samples clustered together in cluster B. In cluster C several *E. coli* isolates from the river (Sand River), dam and irrigation pivot point water (PI 45) clustered together at similarity values above 70%. Cluster D comprised of two *E. coli* isolates from dam water. In cluster E an onion *E. coli* isolate (O33) was 92% similar to water isolate IP 46 from the irrigation pivot point. A second onion isolate (O30) was also grouped in this cluster. The *E. coli* JM109 strain grouped with several dam and river *E. coli* isolates in cluster

F. Furthermore river isolate R36 and dam water sample D42 were 90% similar and both harboured the *stx1* gene. One river and one onion isolate grouped together in cluster G. Cluster H and I was represented by a single *E. coli* isolate from the river and dam respectively. In cluster J one river isolate (R 25) sampled further upstream from the river on the farm, one (R 40) isolated from the same river on the farm and one onion isolate (O23) clustered together at a remarkable similarity value of 92%. These results clearly showed a link between *E. coli* strains from the river to the dam, irrigation pivot point and the onions.

Linking studies with *Escherichia coli* isolates from irrigation water and fresh produce from several study sites

Enterobacterial Repetitive Intergenic Concencus (Eric) PCR fingerprinting

The genetic relatedness of *E. coli* strains from irrigation water from a commercial onion/tomato farm Limpopo), a commercial peach farm (Limpopo) as well as four commercial pear production farms (Western cape) and a limited number of isolates from fresh produce (5 onion, one peach and one pear) was determined using Eric-PCR analysis (Fig. 46). Although *E. coli* was isolated routinely from irrigation water samples, only a few *E. coli* isolates were isolated from vegetables and fruit analysed during this study. These included five onion isolates, two from peaches and one from pears.

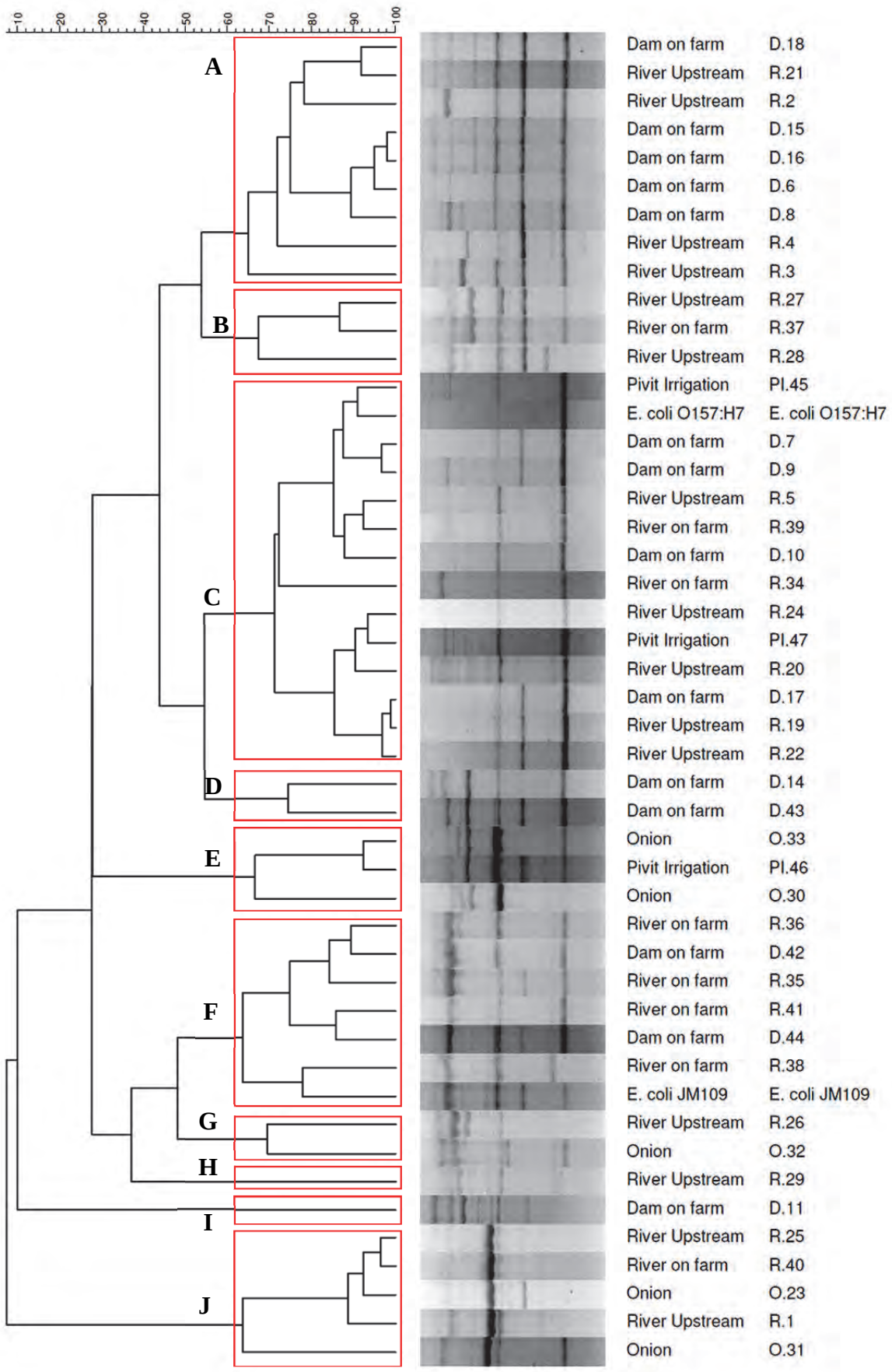


Figure 45: Dendrogram showing the genetic relatedness of *E. coli* isolates from irrigation water and onions.

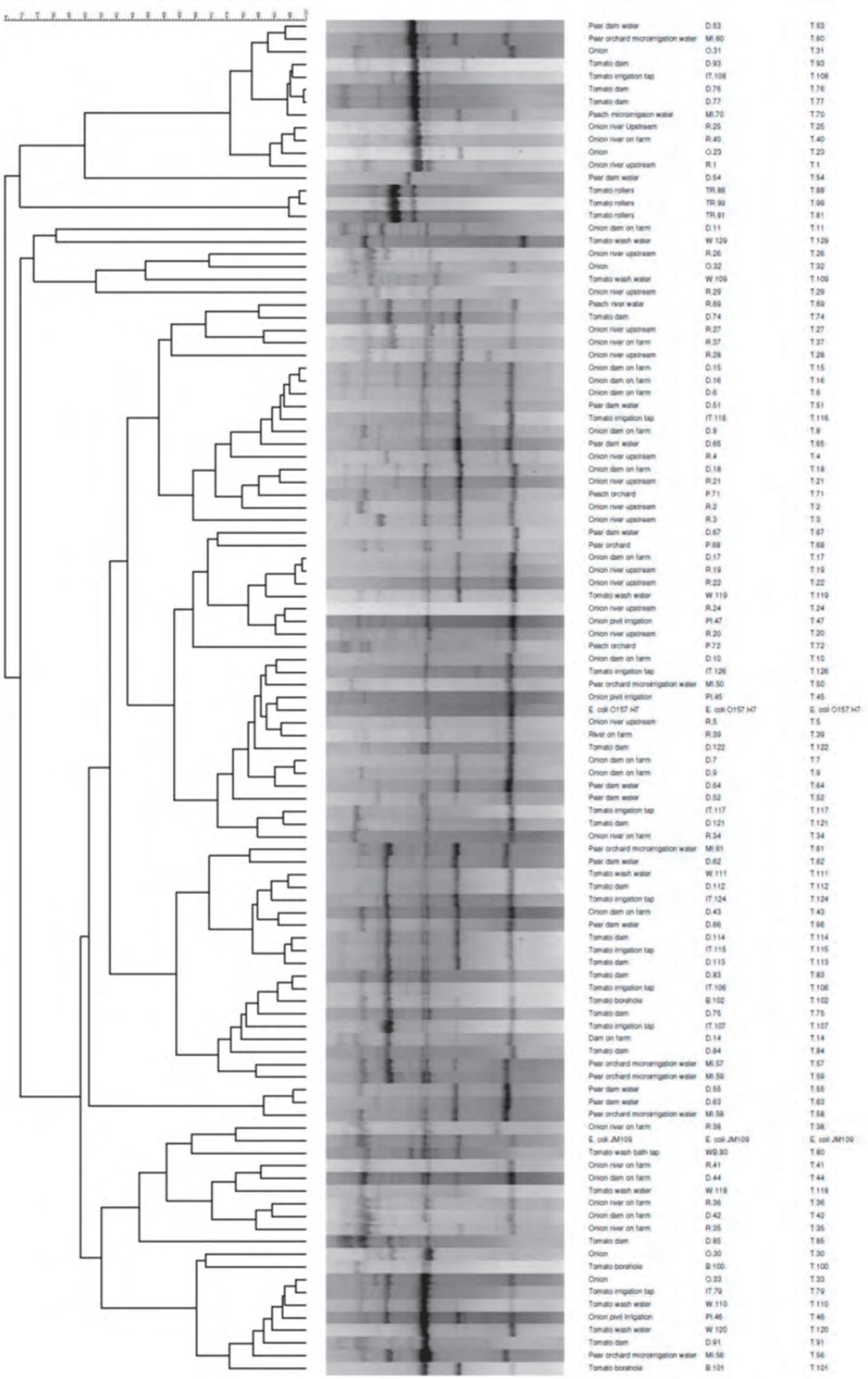


Figure 46: Dendrogram showing the genetic relatedness of *E. coli* isolates from irrigation water sources from several Provinces and associated fresh produce.

Virulence gene analysis

The presence of virulence genes were determined for *E. coli* isolates from irrigation water from a commercial onion/tomato farm (Limpopo), a commercial peach farm (Limpopo) as well as four commercial pear production farms (Western Cape) and a limited number of isolates from fresh produce (5 onion, one peach and one pear) (98 in total). Three isolates (3%) harboured the *eae* gene, five isolates (5%) harboured the *stx1* gene and three isolates (3%) harboured the *stx2* gene. Interestingly one river isolate (R69) from the commercial peach farm had both the *stx1* and *stx2* genes.

Antibiotic resistance of irrigation water, pears and peaches

The *E. coli* isolates from the water samples from the four commercial pear farms showed that the strains were mostly sensitive to the antibiotics (4 in total) listed in Table 68. However, resistance to single antibiotics were detected for some *E. coli* isolates and included either ATM, CAZ or C. Only one isolate was resistant to two antibiotics and included ATM and CAZ. The single pear *E. coli* isolate was also sensitive to all the antibiotics tested. The two *E. coli* isolates from peaches, the river isolate from the commercial farm in Limpopo and the one from the micro irrigation pipes were all sensitive to the antibiotics. A dendrogram of these isolates based on antibiotic resistance profiles relatedness were not constructed since the incidence of antibiotic resistance was extremely low.

Discussion and conclusion

As has been reported for many other rivers in a number of provinces in South Africa, the *E. coli* counts of water from a selected river in the Limpopo River basin (Limpopo Province) frequently exceeded the acceptable levels according to the guidelines of ≤ 1-1000/ 100 mℓ for irrigation water (DWAF 1996) (Gemmell and Schmidt, 2012, 2013; Chigor *et al*., 2013; Aijuka *et al.*, 2015; Teklahaimanot, 2014). Although the *E. coli* counts of the primary irrigation source were unacceptably high, the *E. coli* counts for the dam water exceeded the irrigation water guidelines only once during the sampling period and the water from the irrigation pivot complied with the irrigation water guidelines and is suitable for irrigation purposes. Furthermore no *E. coli* was isolated from any of the onion and tomato samples using coliform/*E. coli* count plates, however *E. coli* was isolated after selective enrichment of the onion samples indicating its presence at levels lower than the standard detectable limit for plate counts.

The decrease observed in the number of total aerobic bacteria, coliforms, fungi and yeasts on the onion surfaces over time during onion production from growth to marketing could possibly be ascribed to 1) known antimicrobial properties of onions against Gram-negative and Gram-positive microorganisms (Hughes *et al*., 1991), and 2) the drying out of the onions in the middens in the field. The reduction in microorganism numbers on onions observed in this study is in agreement with results of another study where populations of *Salmonella* and *E. coli* O157:H7 inoculated with 10^7 CFU/9cm^2 on onions skins, showed a decline of 3 log CFU/g after drying for 30 min (Lieberman *et al.,* 2013).

In this study the aerobic bacterial counts for the tomatoes varied between 1.8 log CFU/g to 3.8 log CFU/g and Salmonella was not detected in any of the tomato samples. Similarly, Gómez *et al.* (2009) reported the mesophilic bacterial counts were lower than 4 log CFU/g on tomatoes. Total aerobic levels of 2 log CFU/g were reported for tomatoes (Cardenas *et al.,* 2013).

Traditionally classical and genomic identification methods were used to identify micro-organisms, however MALDI-TOF MS has been reported to be an alternative method for taxonomical applications (Böhme *et al*., 2011; Dieckmann and von Dohren, 2005). When compared to conventional 16S rRNA gene sequencing, similar results were obtained with MALDI-TOF MS analysis and enabled identification to the subspecies level (De Bruyne *et al*., 2011; Dieckmann and Malorney, 2011). However, to date MALDI-TOF MS has predominantly been used to identify clinical isolates (Ferreira *et al*., 2011; Kroumova *et al*., 2011). A limited number of studies have reported the application of MALDI-TOF MS in a microbial ecological context. The diversity and distribution of cultivable bacteria throughout the whole spinach process line including the fresh produce and the wash water was determined (Hausdorf *et al.,* 2013). In a study to evaluate the microbiological quality of the Msunduzi River in KwaZulu-Natal, South Africa, the identities of *E. coli* isolates was confirmed using PCR and MALDI-TOF MS (Gemmel and Schmidt, 2012). The identification results using the two methods correlated, as was observed in this study. The results of this study confirmed the suitability of MALDI-TOF MS as an alternative method for identification of human pathogenic bacteria isolated from water and fresh produce, comparable to results obtained with PCR analysis.

Phenotypic (antimicrobial) and genotypic (virulence gene prevalence, DNA fingerprinting) characterisation *of E. coli* (including O157:H7) isolates from different sources, i.e. irrigation water, soil, animal and human faeces, meat and lettuce have been used to determine if any of these can act as a source of faecal contamination (Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2011; Aijuka *et al*., 2015; Holvoet *et al*., 2013). Ateba and Mbewe (2014) showed that *E. coli* O157:H7 isolates from a specific farm in South Africa and food product from supermarkets in a particular city or water, grouped together following cluster analysis of Enterobacterial Repetitive Intergenic Concencus (Eric) PCR profiles. Eric PCR analysis clearly grouped some of the river, dam, irrigation pivot point and onion samples at similarity values ranging between 80% and 92% which showed the link between the irrigation water sources and the onion isolates.

Resistance to multiple antimicrobial agents in microorganisms was observed for *E. coli* isolates, isolated from irrigation water sources and onions, might have been due to the river being downstream of two municipal sewage works close to an urban area in several similar studies in South Africa (Olaniran *et al*., 2009; Da Silva *et al.,* 2011; Aijuka *et al.,* 2015). Results of this study confirmed that irrigation water sources can act as reservoirs of antibiotic resistant microorganisms and poses a food safety and health risk.

3.2.9 LIMPOPO PROVINCE – Potential link between irrigation water quality and the microbiological quality and safety of peaches from a commercial peach farm near Mookgophong

Specific aim

The aim of this study was to investigate the microbiological quality of peaches and water used for irrigation purposes with the ultimate aim of establishing whether a link between the irrigation water and fresh fruit can be made.

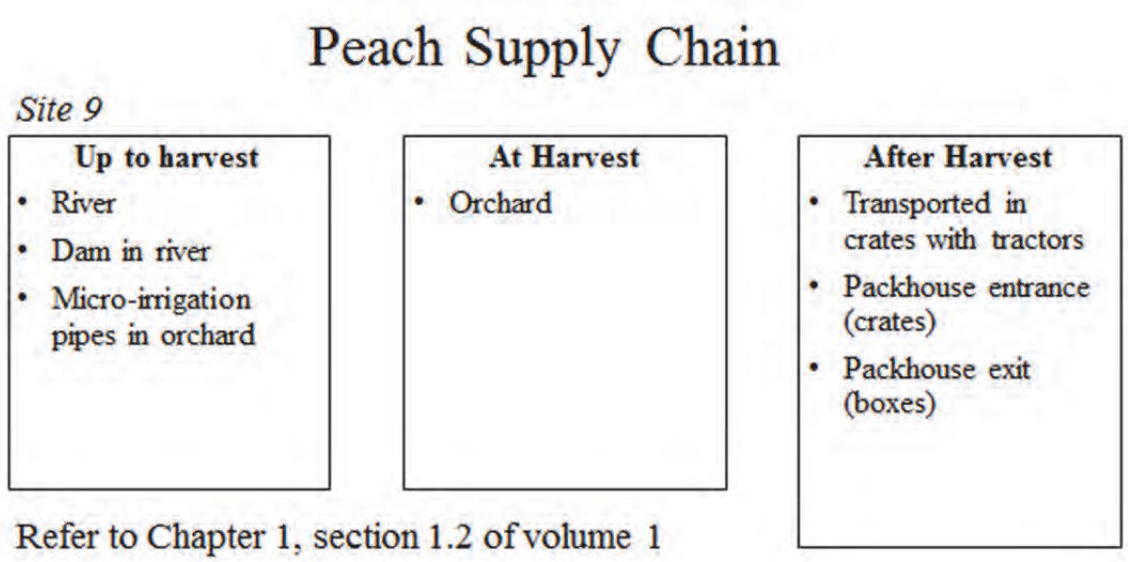
Experimental procedures

Experimental site

- The study was conducted on a Global GAP (Good Agricultural Practices) certified commercial farm. Peaches (Cultivar=Sunette) were sampled from a peach farm located in the Mookgophong (Naboomspruit) area in the Northern Province, South Africa.
- The peach farm is Global GAP (Good Agricultural Practices) certified and has a traceability system in place.
- Water from the Sterk River and dams in the river are used for irrigation.
- Peaches were irrigated by use of micro irrigation and pivots.
- For the sake of our study, a single orchard (HH) on the farm was selected for microbiological assessment of peaches.

Process flow

After harvesting in the orchard, the peaches are packed into plastic crates and transported on a tractor to the packhouse. Upon receiving at the packhouse, the peaches are sorted according to size using a size selector line and packed by hand where after it is dispatched in cooled trucks to the fresh produce market in Johannesburg.



Sampling
Description of water sampling sites

Four water sampling sites linked to the orchard (Orchard HH) where the peaches were harvested were chosen for determining the microbiological quality and safety of the irrigation water. These included water from the Sterk River, dam in the river, irrigation pipe water after filters in the field and from micro irrigation pipes.

Peach and water sampling and processing

Water samples (100 mℓ, 1 ℓ and 10 ℓ respectively) were collected and microbiological analysis was performed as described in section 3.2.1, for water used to irrigate parsley.

From the orchard five batches of eight fruit each were sampled from the crates on tractors arriving at the packhouse. Five batches of eight fruit each were sampled from the boxes after packing. For all samples, microbiological analysis was conducted within 24 hours after sampling.

Sonication
Each batch of peaches was transferred into a beaker containing 1liter Ringers' solution with 0.02% Tween 80, the displacement volume noted and sonicated in a Digital Ultrasonic Cleaner (EUmax®, Labotec, Model no. UD200SH-6L) for 30 seconds. After removal of the peaches, the solution was filtered, using 0.45µm filter membranes.

Microbiological analysis
Total aerobic colony counts, coliform/*E. coli*, fungi and yeast counts were determined as described for onions (section 2.3.8). Slanetz and Bartley medium (Oxoid) supplemented with

2,3,5-triphenyltetrazolium chloride (TTC solution, Oxoid) and Staph Express count system (3M Petrifilm) were used for the enumeration of Intestinal enterococci (IE) and *S. aureus* respectively. Colonies were enumerated after incubation of plates at 37°C for 24-48 h. Presumptive intestinal enterococci colonies were transferred onto Bile Esculin Azide Agar (Oxoid) and incubated at 44°C for 2 h (ISO 7899-2:2000). Enrichment for the detection of *Escherichia coli* O157: H7, *Salmonella* Typhimurium and *Listeria monocytogenes* were done as described in section 2.3.1. Identities of single colonies of presumptive positive colonies isolated from chromogenic media were confirmed using MALDI-TOF analysis (see section 2.3.1).

Results

Water

Aerobic colony counts (ACC) – Total average bacterial counts were higher after filters installed in irrigation pipes in the orchard and in micro irrigation pipes in the orchard when compared to river and dam water counts (Table 76).

Yeasts – Total average yeast counts were higher after filters installed in irrigation pipes in the orchard and in micro irrigation pipes in the orchard when compared to river and dam water. The highest yeast count was 4.49 log CFU/mℓ (Table 76).

Fungi – The average fungi counts ranged between zero and 1.65 log CFU/mℓ (Table 76).

Coliforms and E. coli – The *E. coli* levels in the irrigation water (Sterk River, dam, micro irrigation pipes in the orchard and filters in the irrigation pipes) ranged between 3.2 log CFU/100 mℓ and 4 log CFU/100 mℓ and exceeded the DWAF and WHO guideline limit of 1000 CFU/100 mℓ (Fig. 47).

Enterococcus – Counts were very low ranging from 0.5 log CFU/mℓ to 1 log CFU/mℓ.

E. coli O157 (including H7) **–** none isolated.

Salmonella – none isolated.

Listeria spp. – none isolated.

Table 76 Summary of results obtained for microbiological analysis of irrigation water from a commercial peach farm

Irrigation water source	Average microbial counts Log CFU/mℓ				Average microbial counts Log CFU/100mℓ	
	Aerobic bacteria (ACC)	Fungi	Yeast	Entero-coccus	*E. coli*	Coliforms
River	2.93 (0.22)	0.4 (0)	2.51 (0.17)	0.94 (0)	5.42 (0.14)	6.54 (0.22)
Dam	3.14 (0.22)	1 (0)	2.09 (0.41)	1 (0)	5.47 (0.04)	7.01 (0.18)
Pipe in orchard after filter	4.63 (0.95)	0.00 (0)	4.49 (0.92)	1.33 (0)	5.24 (0.18)	7.56 (0.63)

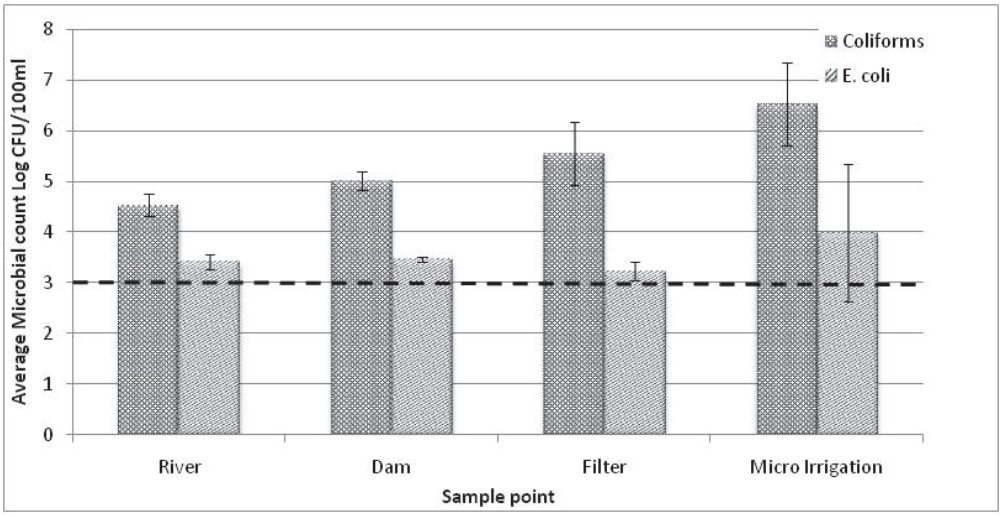


Figure 47: Coliform and *E. coli* counts of irrigation water sources used to irrigate peaches. The broken line indicates the South African Department of Water Affairs irrigation water guideline limit of 1 000 *E. coli* CFU/100 mℓ (DWAF, 1996).

Peaches

Aerobic colony counts (ACC) – The results showed that the aerobic plate counts for the peach surfaces from the orchard, crates entering the packhouse and in boxes after packing were similar, ranging from 4-4.5 log CFU cm^2 (Table 77).

Coliforms and E. coli – Coliform counts were similar ranging from 1.6 log CFU/ cm^2 in the orchard to 1.3 log CFU/ cm^2 in boxes after packing (Table 77). *E. coli* was isolated from orchard samples only at < 1 log CFU/ cm^2.

Enterococci – counts of 0.22 log CFU/ cm^2 on the surfaces of peaches from the orchard. None were detected on peaches in the crates entering the packhouse or after packing (Table 77).

Yeasts and fungi – Yeast numbers were similar to the total aerobic bacterial counts ranging between 4 and 4.5 log CFU/ cm^2 (Table 77). Fungi were detected on peaches from the orchard at 0.7 log CFU/ cm^2 and not on packhouse peaches.

E. coli O157 (including H7) **–** none isolated.
Salmonella – none isolated.
Listeria spp. – none isolated.

The dominant bacteria identified on the peach surfaces were *Pantoea agglomerans, Bacillus cereus, Enterobacter cowanii* and *Enterococcus hirae*. Dominant fungi isolated from MEA medium were identified as *Fusarium* spp., *Alternaria* spp. and *Epicoccum nigrum*.

Table 77 Summary of results obtained for the microbiological analysis of peaches

Produce, sites	**Average microbial counts log CFU/ cm^2**				
Peaches	**Aerobic bacteria (ACC)**	**Fungi**	**Yeast**	**Petrifilm**	**Enterococci**
Orchard	3.80 (0.19)	1.81 (0.09)	3.91 (0.2)	1.38 (0.17)	0.31 (0.02)
Crates Entrance)	4.16 (0.21)	0.00	4.00 (0.2)	1.19 (0.06)	0.43 (0.02)
Packhouse Exit	3.55 (0.18)	0.00	3.74 (0.19)	0.99 (0.05)	1.01 (0.05)

Discussion and conclusion

The *E. coli* levels in the irrigation water (Sterk River, dam, micro irrigation pipes in the orchard and filters in the irrigation pipes) exceeded the DWAF and WHO guideline limit of 1000 CFU/100 mℓ. Total average bacterial and yeast counts were higher for samples taken from irrigation pipes after filters as well as in micro-irrigation pipes in the orchard when compared to river and dam water counts. This could indicate an accumulation of microorganisms as opposed to improving the water quality by installing filters in the irrigation pipes in the peach orchard. None of the human pathogenic bacteria tested for (*E. coli* O157 (including H7), *Salmonella* spp. and *Listeria* spp. were isolated from the water samples. The microbiological quality of the peaches was mostly acceptable according to DoH guidelines. *E. coli* was detected on orchard peaches only, *E. coli* O157 (including H7), *Salmonella* spp. and *L. monocytogenes* were not detected in any of the fruit samples. The peaches can generally be regarded as acceptable for human consumption based on these criteria.

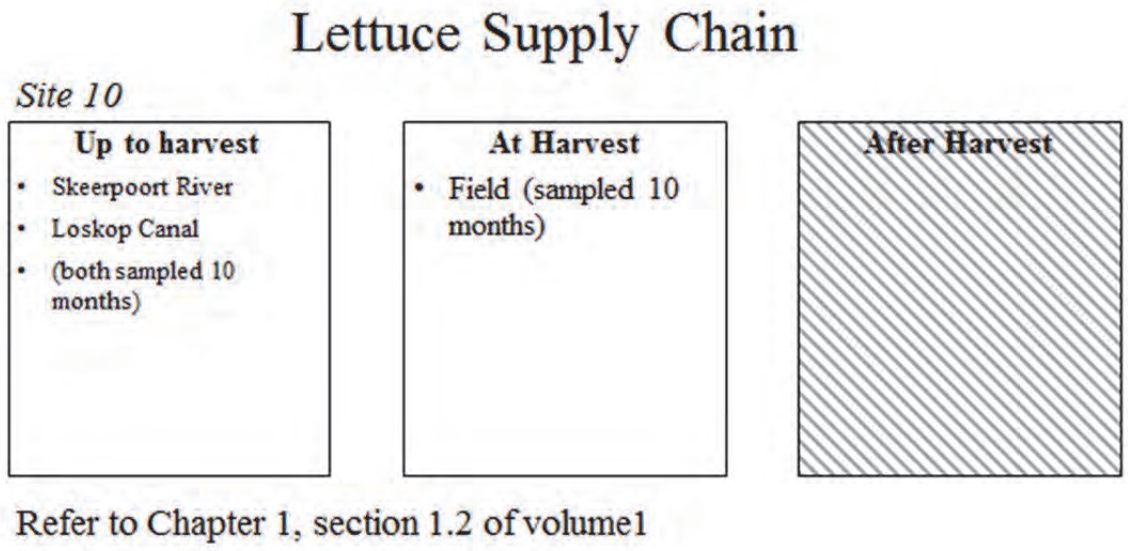
3.2.10 NORTH WEST PROVINCE – Potential link between the microbiological quality of irrigation water and the microbiological quality and safety of lettuce from a farm near Skeerpoort

Specific aim: The aim of this study was to determine whether irrigation water from the Skeerpoort River located in the North West Province of South Africa was a source of foodborne bacterial pathogens on irrigated lettuce over two seasons (10 months).

Experimental procedures

Site Selection

Irrigation water was collected from the Skeerpoort River (North West Province). The Skeerpoort River is downstream of the Hartbeespoort Dam, a major tourist location surrounded by an urban settlement. A vegetable growing farm using water from a dam containing water diverted from the Skeerpoort River for irrigation purposes was chosen as the sampling site for lettuce.



Sample Collection

Water: One water sample was aseptically collected (SANS, 2006) on a monthly basis the Skeerpoort River for 10 months from January to October, 2011.

Lettuce: Crisphead lettuce samples were collected monthly at harvest from a field irrigated with water from the Skeerpoort River for 10 months from January to October 2011.

Physico-chemical parameters

Temperature (at sampling site) and pH (on arrival at the laboratory) (Check temp 1, Hana instruments Inc, Rhode Island, USA) for each water sample were measured.

Microbiological analysis

Bacterial indicators (Aerobic colony counts, ACC; Anaerobic spore formers, AnSF; Aerobic spore formers, ASF; Intestinal enterococci, IE; Total coliforms, TC; Faecal coliforms, FC) and pathogens (*E. coli*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella* spp.) were enumerated and identified (Table 78).

Characterization and identification of aerobic bacteria

For each lettuce sample analysed, 3 colonies were randomly picked from the highest dilution standard plate count agar petri-dish. Gram stain and catalase tests were done. Thirty (30) bacterial isolates were used. 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, Oklahoma, USA, 2011) to test for significant differences in microbiological quality at 95% confidence interval between sampling intervals.

Table 78 Methodology for detection of bacterial organisms on lettuce

Organism	Media	Temperature/ Time of incubation	Company	Reference
Aerobic colony count (ACC)	Standard Plate Count agar	30°C for 48 to 72h	(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, 1998)
Anaerobic spore formers (AnSF)	Trypticase Soy Agar	35°C for 48 h		
	Lauryl Tryptose (LST) Broth	35°C for 24 to 48h	(Oxoid Ltd, Basingstoke Hampshire, UK)	MFHB19 (Health Canada, 2002)
Total coliforms	Brilliant Green Bile 2% Broth (BGLB)			
Faecal coliforms	EC Broth			
E. coli	Eosin Methylene Blue Agar (Levine) (EMB)	35°C for 24h		
	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)	
Intestinal enterococci	Slanetz and Bartley Medium	35°C for 44h	(Oxoid Ltd, Basingstoke Hampshire, UK)	(SANS ISO 7899-2) (2004)
	Bile Esculin Agar	44°C for 2h		
S. aureus	Baird-Parker Agar	35°C for 48h	(Oxoid Ltd, Basingstoke Hampshire, UK)	SABS ISO 6888-1 (1999)
§*Salmonella* spp	Brilliance ™ Salmonella Agar Base	35°C for 24 to 48h	(Oxoid Ltd, Basingstoke Hampshire, UK)	SABS ISO 6579 (2003)
	Rapid Salmonella	35°C for 24 to 48h	BioRad	
†*L. monocytogenes*	Listeria Selective Agar (Oxford formulation)	35°C for 48 h	(Oxoid Ltd, Basingstoke Hampshire, UK)	SABS ISO 11290-1 (1996)
	Palcam Selective Supplement			

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

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

Linking studies:

Antimicrobial testing

Twelve *E. coli* isolates from the Skeerpoort River and ten isolates from lettuce irrigated with water from the Skeerpoort River were used in the study.

Antibiotics used included; Amikacin (30 µg), Gentamicin (10 µg), Chloramphenicol (30 µg), Nalidixic Acid (30 µg), Norfloxacin (10 µg), Neomycin (30 µg), Nitrofurantoin (300 µg), Amoxicillin (25 µg), Ampicillin (10 µg), Cephalothin (30 µg) and Oxytetracycline (30 µg). Antibiotic susceptibility testing was determined by disc diffusion method using Mueller-Hinton agar (Oxoid).

Virulence gene testing

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, Hercules, California). The thermocycler (C1000 Touch ThermalCycler CFX96™ 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).

Genetic fingerprinting

$(GTG)_5$ Rep-PCR was carried out as reported by Aijuka *et al*. (2015). The same isolates used for antibiotic resistance were used**.** DNA preparation was performed using ZR Fungal/Bacterial DNA Kit™ (Zymo Research, Irvine, California) from single colonies. The 20 µℓ reaction consisted of 17.6 µℓ, 1.1 times HotStarTaq Plus Master Mix Kit (Qiagen, Limburgh, Netherlands), 0.2 µM $(GTG)_5$ primer, 1.6 µℓ DNA template and 1% dimethylsulphoxide. Amplification was carried out as follows: initial denaturation at 95° C for 10 min; 35 cycles of 95°C for 30 s, 40°C for 60 s and 65°C for 3 min; and a final elongation step at 65°C for 8 min. The PCR amplified DNA was separated on 1.6% agarose gel containing SYBR® safe gel (Invitrogen, Carlsbad, California) by electrophoresis and visualized on the UVTEC Cambridge image (Bio-Rad) under UV light. The molecular marker 1500bp (Invitrogen) was used to estimate the length of amplicon isolates from irrigation water and lettuce.

Statistical analysis linking studies

Analysis of variance was performed to test for significant differences in antibiotic resistance patterns for *E. coli* isolated from The 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). Fingerprints from $(GTG)_5$-Rep-PCR were analysed using GelCompare III version 5.10 (Applied Maths, Saint-Marten-Latem Belgium). Similarity among digitized bands was calculated using Pearson correlation, and an average linkage (UPGMA or unweighted pair group method with

arithmetic averages) dendrogram was derived from the profiles. Full cluster formation was defined at points where complete nodes were formed.

Results

Water:

Microbiological analysis of irrigation water

Aerobic colony counts (ACC) – the ACC in irrigation water ranged between 2.6 log CFU/mℓ and 4.6 log CFU/mℓ (Fig. 48).

Anaerobic spore formers (AnSFs) – ranged between zero and a maximum count of 5.2 log CFU/ mℓ (Fig. 48).

Aerobic spore formers (ASF) – ranged between zero and a maximum count of 4.6 log CFU/ mℓ (Fig. 48).

S. aureus – the counts ranged between zero and a maximum of 2 log CFU/ mℓ (Fig. 48).

Faecal coliforms (FC) – FC counts varied between zero and a maximum of 3.8 log MPN/ 100 mℓ.

E. coli **–** *E. coli* was present during 4 of the 10 sampling intervals (Fig. 48).

Salmonella spp. – absent

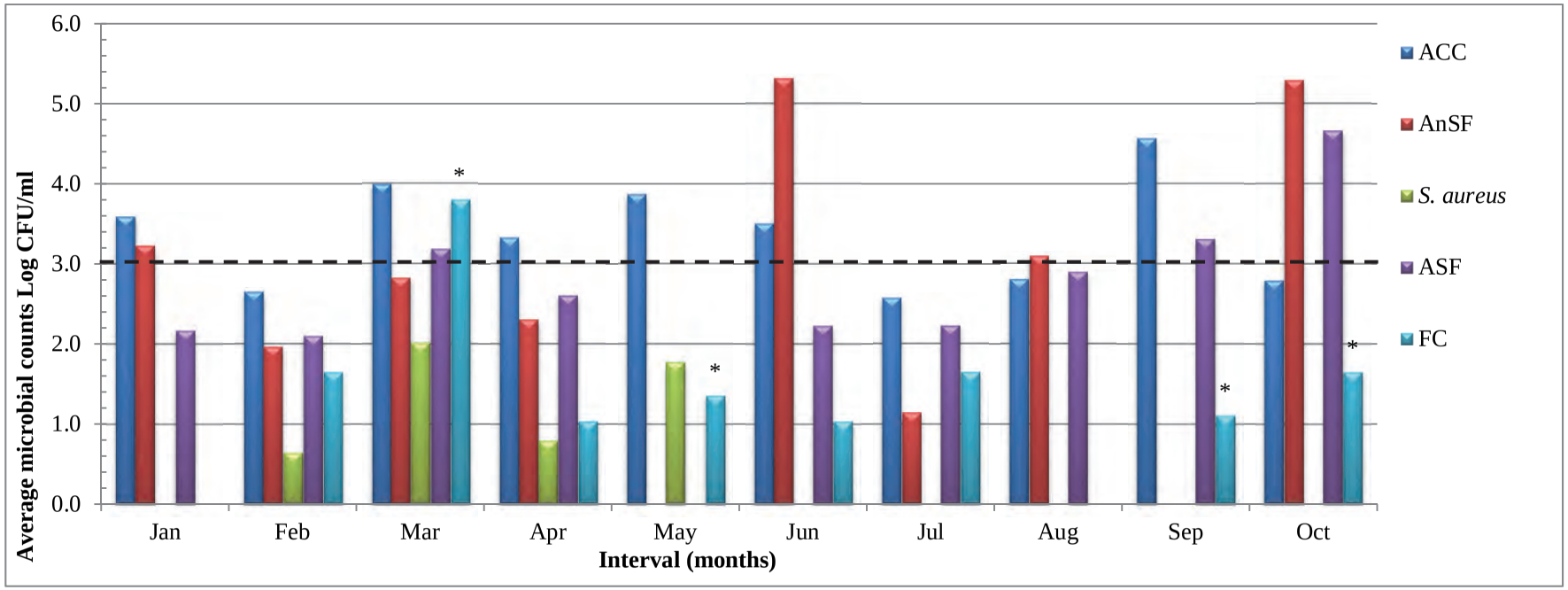


Figure 48: Change of bacterial counts and incidence of *E. coli* with time in irrigation water from the Skeerpoort River for samples (n=10) collected monthly over 10 months. (*) Interval positive for *E. coli*. Aerobic colony count (ACC), Anaerobic spore formers (AnSF), Aerobic spore formers (ASF), *Staphylococcus aureus* (*S. aureus*) and Faecal coliforms (FC: (log MPN/100 mℓ). Horizontal line represents upper limit for faecal coliforms in irrigation water (DWAF, 1996).

Physicochemical quality indicators of water from the Skeerpoort River

The pH ranged from 7.9 to 9.2 in the Skeerpoort River. The pH in the Skeerpoort River was acceptable according to the South national guidelines (DWAF, 1996) for irrigation water twice during the 10 month study period. Water temperature in the Skeerpoort River ranged from 10.7 to 26.0˚C with the lowest and highest noted in May and January respectively.

Microorganism identification

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. *E. coli* was most prevalent in winter and *Bacillus* spp. in summer in the Skeerpoort River. Low prevalence (3.3%) of *Salmonella* spp., *Klebsiella* spp., *Raoultella* spp., *Burkholderia* spp., *Buttiauxella* spp. was noted. Prevalence of predominant bacteria from lettuce irrigated with water from the Skeerpoort River for isolates (n=30) collected monthly over 10 months, are summarised in Table 79.

Lettuce:

Aerobic colony counts (ACC) – The maximum ACC noted was 7.3 log CFU/g lettuce during August and the lowest during June and July, i.e. 4.9 and 5.1 log CFU/g respectively (Fig. 49). There was a significant difference ($p \leq 0.05$) in ACC over the different months.

Anaerobic spore formers (AnSFs) – the maximum count was 5.5 log CFU/g and the minimum 1 log CFU/g (Fig. 49).

Aerobic spore formers (ASF) – the counts ranged between maximum 4.3 log CFU/g and minimum 3 log CFU/g and were present at all the sampling intervals (Fig. 49).

S. aureus – the counts ranged between 3.9 log CFU/g (maximum) and 1.5 log CFU/g (minimum and were present during 9 of the 10 sampling intervals (Fig. 49).

Faecal coliforms (FC) – FC counts varied between 2.8 log CFU/g and 3.5 log CFU/g were present during 5 of the 10 intervals (Fig. 49).

E. coli **–** *E. coli* was present during 3 of 10 sampling intervals (Fig. 49).

Salmonella spp. – absent

L. monocytogenes – present

Irrigation water and lettuce: The microbial counts and percentage of water and lettuce samples (n=20) positive for pathogens for samples collected between January and November 2011 from the Skeerpoort River and adjacent lettuce field are summarised in Table 80.

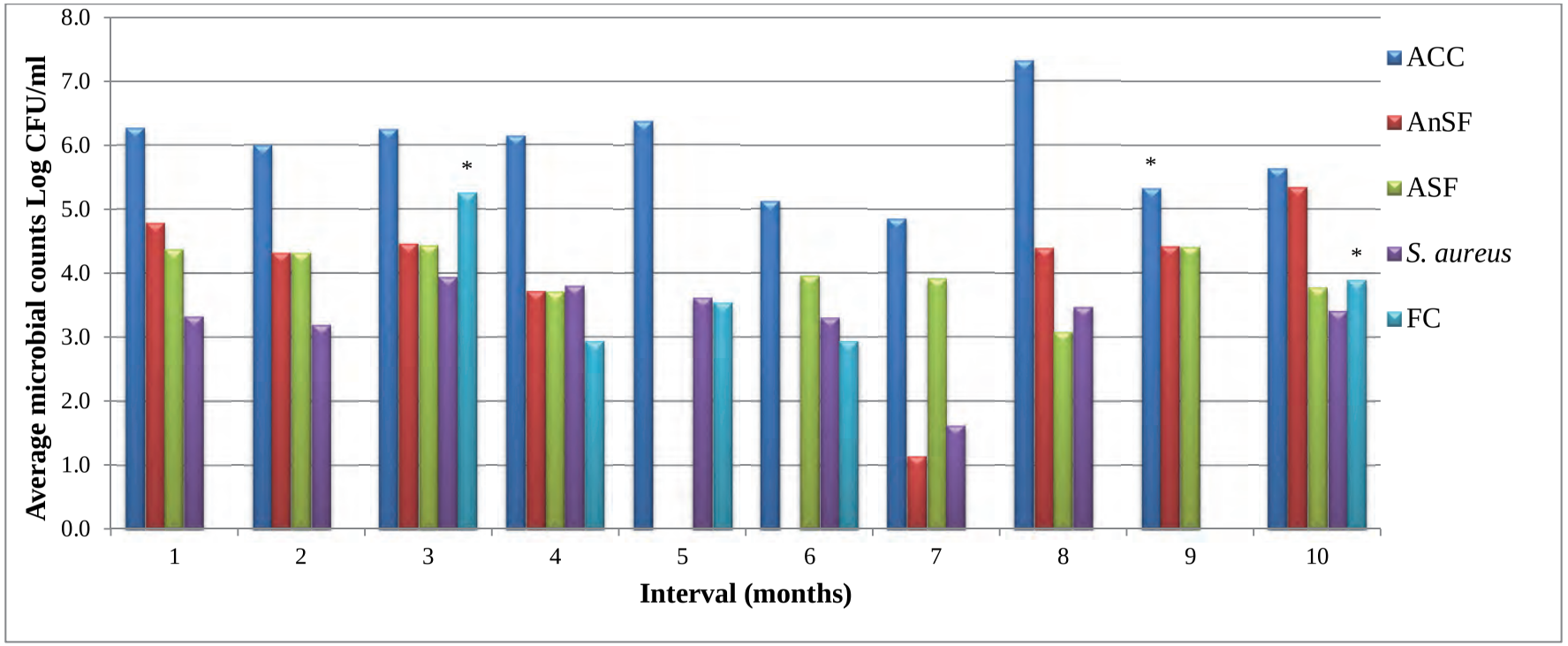


Figure 49: Change in bacterial counts and incidence of *E. coli* on lettuce irrigated with water from the Skeerpoort River for samples (n=10) collected monthly over 10 months. (*) interval positive for *E. coli*. Aerobic colony count (ACC), Anaerobic spore formers (AnSF), Aerobic spore formers (ASF), *Staphylococcus aureus* (*S. aureus*) and Faecal coliforms (FC).

Table 79 Prevalence of predominant bacteria from lettuce irrigated with water from the Skeerpoort River for isolates (n=30) collected monthly over 10 months

Month	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Total number of isolates**
Percentage isolates											
E. coli	–	–	33	–	67	67	33	–	100	67	22
Bacillus spp	67	33	–	33	33	–	33	100	–	–	17
Enterobacter spp	–	33	33	–	–	–	–	–	–	–	7
Klebsiella spp	–	–	33	–	–	–	–	–	–	–	3
Enterococcus spp	–	–	–	–	–	–	33	–	–	–	1
Salmonella spp	–	–	–	–	–	–	–	–	–	–	1
Serratia spp	–	–	–	–	–	–	–	–	–	33	1
Raoultella spp	–	–	–	–	–	33	–	–	–	–	2
Leclerciaa decarboxylata	33	–	–	–	–	–	–	–	–	–	1
Brevibacillus borstelensis	–	–	–	33	–	–	–	–	–	–	1
Exiguobacter iumundae	–	–	–	33	–	–	–	–	–	–	1
Staphylococcus kloosi	–	33	–	–	–	–	–	–	–	–	1
Bautiaxella agrestis	–	–	–	–	–	–	–	–	–	–	1
Burkholderia caprophylil	–	–	–	–	–	–	–	–	–	–	1
Total number of isolates	3	3	3	3	3	3	3	3	3	3	60
ACC (log CFU/mℓ)	6.3	6.0	6.3	6.1	6.4	5.1	4.9	7.3	5.3	5.6	

– Not determined ACC – aerobic colony counts.

Irrigation water and lettuce

A comparison of the ACC counts for lettuce and irrigation water over the ten month period can be viewed in Figure 50. Statistical analysis performed between the ACC counts of the Skeerpoort River and lettuce showed a significant difference ($p \leq 0.05$). Microbial counts and percentage of water and lettuce samples (n=20) positive for pathogens for samples collected from the Skeerpoort River and adjacent lettuce field are summarised in Table 80.

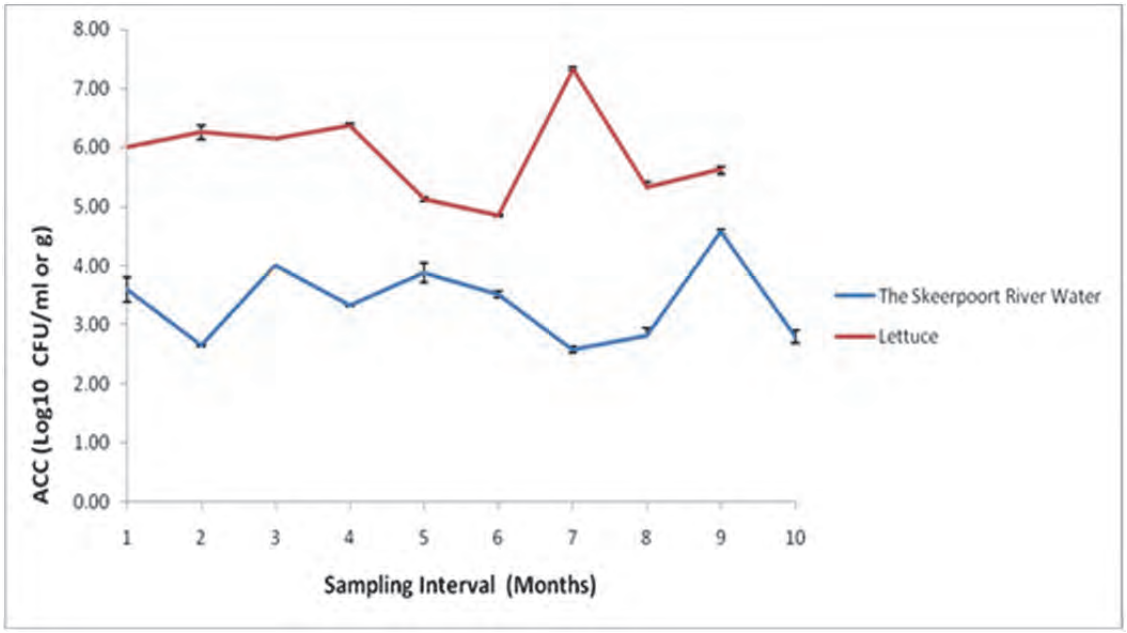


Figure 50: Aerobic Colony Counts of the Skeerpoort River and Lettuce (n=20).

Table 80 Summary of microbial counts and percentage of water and lettuce samples (n=20) positive for pathogens for samples collected between January and November 2011 from the Skeerpoort River and adjacent lettuce field.

Sample	No. of samples	Indicator parameters				% of samples positive for pathogens			
		Aerobic Colony Count (ACC)	Anaerobic spore formers AnSF	Aerobic spore formers ASF	Faecal coli-forms	*Salmo-nella* ssp.	*Liste-ria* ssp.	*E. coli*	Other Micro-organisms
		Log CFU/mℓ			Log CFU/100 mℓ				
Skeer-poort River water[1]	10	3.4±0.7 [a]	2.5± 1.8 [a]	2.5±1.2 [a]	1.3 ±1.1	0	0	40	70
Lettuce [2]	10	5.9±0.7 [b]	3.3±2.0 [b]	3.6±1.9 [b]	1.9±2.1	0	30	30	100
Average	20	4.7±1.5	2.9± 1.9	3.1±1.4	1.6±0.4	0	15	35	85

Linking studies:

Antibiotic resistance 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 which isolates in the Skeerpoort River were additionally resistant to (Table 81). 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 81).

Table 81 Prevalence of antibiotic resistant *E. coli* collected over 10 months from the Skeerpoort River and lettuce irrigated with water from the Skeerpoort River

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

- 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 81).

Three antibiotic resistance patterns were noted among isolates from lettuce (Table 82). More isolates from the Skeerpoort River showed resistance to multiple antibiotics (Table 82). Most isolates from the same sample showed closer phenotypic relatedness based on resistance to the antibiotics compared to those from different samples (Fig. 51). 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 (Fig. 51).

Cluster analysis using $(GTG)_5$-Rep PCR generated five distinct clusters (A, B, C, D and E) (Fig. 52). Some *E. coli* isolates from both sources showed complete (100%) genetic relatedness. There were 33% of isolates from irrigation water having 100% genetic similarity to 60% of isolates from lettuce.

Table 82 Multiple resistances to antibiotics in *E. coli* collected over 10 months from the Skeerpoort River and lettuce irrigated with water from the Skeerpoort River

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

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

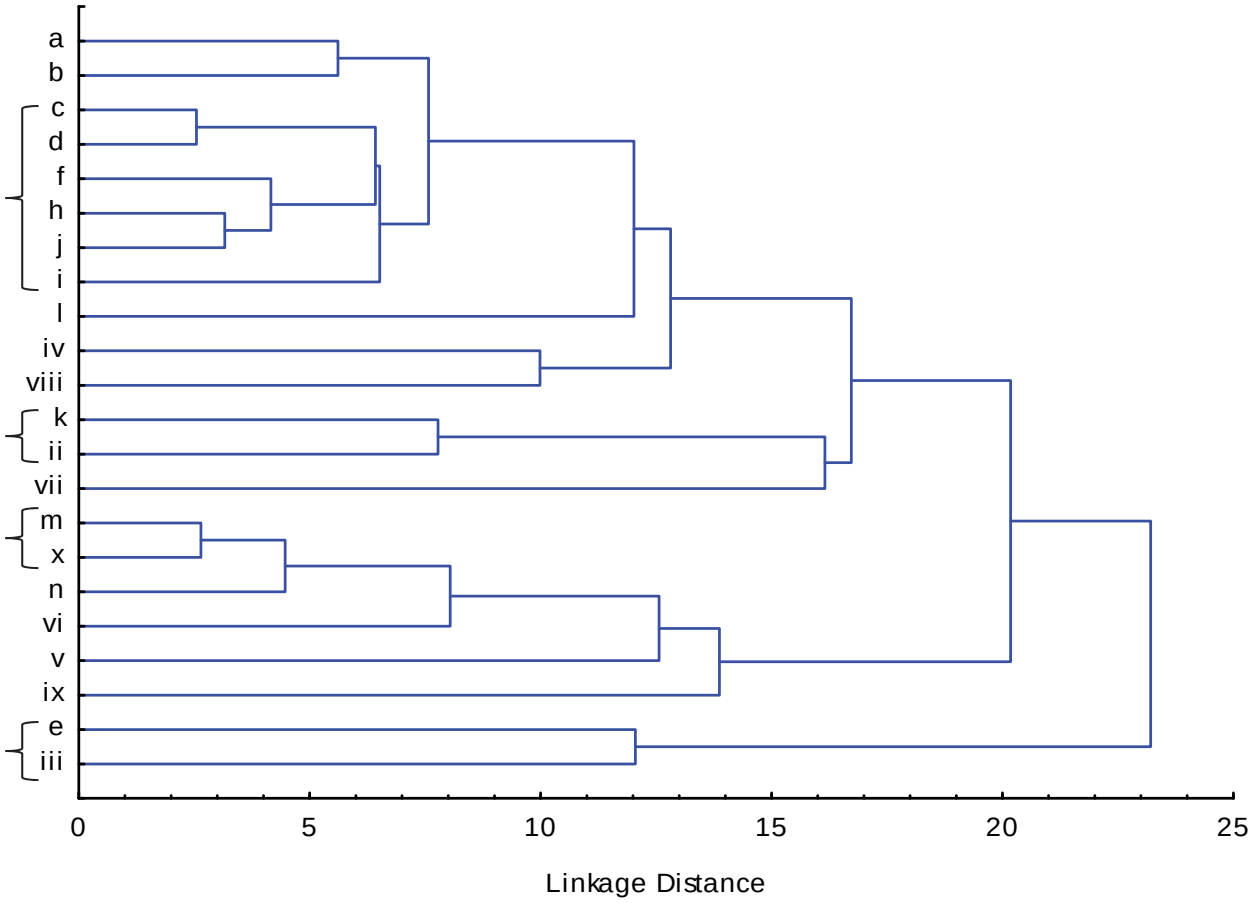


Figure 51: Dendrogram showing cluster formation for *E. coli* collected over 10 months from the Skeerpoort River and lettuce irrigated with water from the Skeerpoort River. Alphabetical letters represent isolates from the Skeerpoort River while roman numerals represent isolates from lettuce. The isolate 'i' is a Roman numeral. Brackets enclose closely related isolates from the two samples

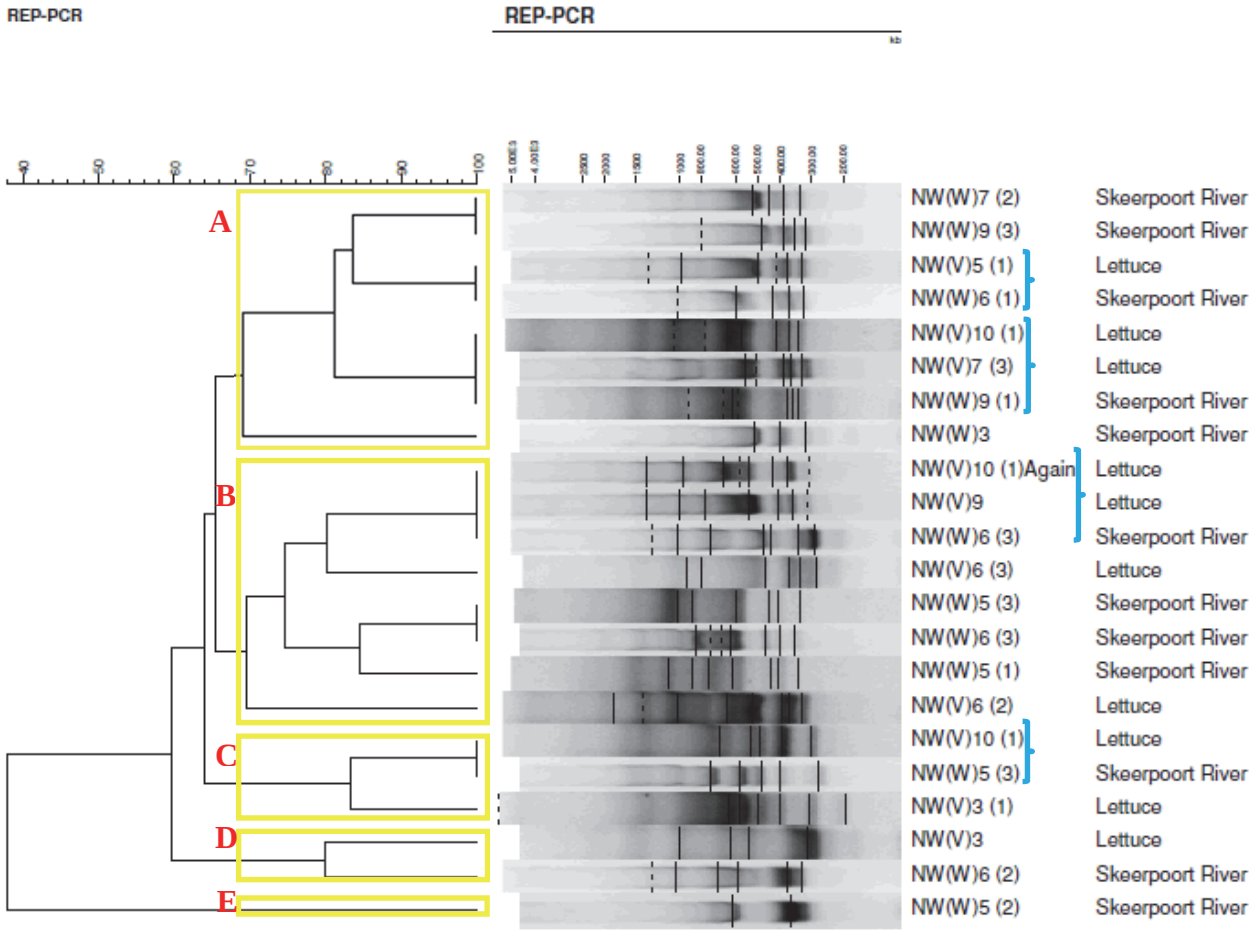


Figure 52: Dendrogram showing $(GTG)_5$-PCR fingerprints of *E. coli* collected from the irrigation water (12) and irrigated lettuce (10). Brace brackets enclose isolates from irrigation water and lettuce that showed 100% genotypic similarity. Isolate codes are directly opposite the source of isolation. Brace brackets enclose isolates from irrigation water and lettuce that showed 100% genotypic similarity. Isolate codes are directly opposite the source of isolation. A, B, C, D and E indicate definite clusters made up of *E. coli* isolates from irrigation water and lettuce.

Discussion and conclusion

The high Aerobic colony counts (ACC) on lettuce is probably due to the fact that lettuce grows in close proximity to soil which contains high levels of bacteria. Additionally, fresh vegetables have large numbers of bacteria growing as part of their normal flora (Gilbert *et al*., 2000). In a recent study ACC in irrigated fresh vegetables ranged from 4.3 to 10.4 log 10 CFU (Halablab *et al*., 2011) and 3 to 4 log CFU/g (Ijabadeniyi, 2010). Contamination of lettuce with anaerobic and aerobic spores could also possibly be attributed to soil, considered a major reservoir of microorganisms. Similarly, Ijabadeniyi (2010) reported low counts of aerobic and anaerobic spores on broccoli and cauliflower irrigated with water from the Loskop canal.

The presence of *L. monocytogene*s on the lettuce indicates contamination either from the water source or the soil it is cultivated in. Fertilization of soil with manure has been shown to increase the risk of contaminating produce with *Listeria* spp. because of its high concentrations (Ackermann, 2010). *L. monocytogenes* present in soil with manure can move onto the vegetables staying viable for a long time. Additionally, *L. monocytogenes* was also not recovered from cauliflower irrigated with water containing the pathogen (Ijabadeniyi, 2011). This indicates that irrigation water may not have been a notable source of the pathogen on lettuce. Presence of *L. monocytogenes* on lettuce is of concern because it was associated with a foodborne outbreak associated with cantaloupes in the United States leading to a recall of produce and fatalities (Centers for Disease Control and Prevention (CDC) 2011).

The ability of *E. coli* to proliferate on lettuce could have been enhanced by the large surface area of its leaves and foliar surfaces with many folds and fissures, which provided good shelter for the pathogen (Halablab *et al*., 2011). Additionally bruises and cuts on vegetables could have been possible points of attachment for bacterial pathogens because slight abrasions on leaves from processes such as dropping irrigation water can cause microscopic damage which can facilitate pathogen proliferation (Aruscavage, 2007). Furthermore moisture and exudates from plant material through bruises and cuts leach nutrients that promote microbial growth (Ampadu, 2007).

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). High multiple resistances to antibiotics within *E. coli* isolated from the Skeerpoort River may be linked to the existence of a large pool of antibiotic resistance bacteria.

Rivers such as the Skeerpoort River, flowing through urban areas, may have increased exposure to higher levels of pollution attributed to domestic and industrial waste (Walters *et al*., 2011). This increases the risk of contamination with antibiotic resistant bacterial pathogens that may be transferred on to irrigated produce such as lettuce. 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 can serve as reservoirs of antibiotic resistant pathogens posing a food safety and public health threat. 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). 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. Additionally

similar genetic relatedness shown by some isolates from irrigation water and lettuce further implicates irrigation water as a source of pathogenic bacterial contamination.

Results from this study show that surface irrigation water sources in South Africa can be reservoirs of antibiotic resistant *E. coli*. Additionally, irrigation water with poor bacteriological quality may contaminate irrigated produce, potentially presenting a risk to both food safety and public health.

3.2.11 NORTH WEST – Potential link between irrigation water quality (irrigation channel fed by Crocodile River and Hartbeespoort Dam) and microbiological quality and safety of Crisp lettuce from harvesting to end of shelf life in the Brits area

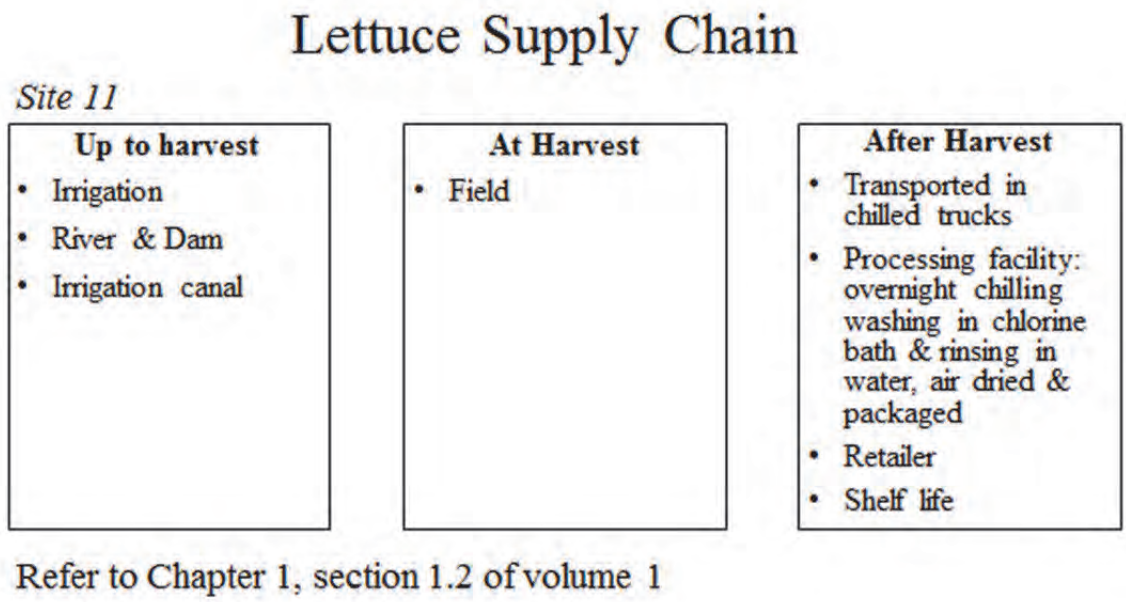
Specific aim

The objectives of this study were to evaluate the microbiological quality of irrigation water and field lettuce from planting to end of shelf life. Additionally, the antimicrobial susceptibility of selected intestinal enterococci isolated from field lettuce samples were determined.

Experimental procedures

Experimental site

- The lettuce farm is located in the Brits area in the North West province, South Africa.
- The lettuce farm is Global GAP (Good Agricultural Practices) certified and has a traceability system in place.
- Water from the Crocodile River and Hartbeespoort Dam feeds the irrigation canal channelled to the farm for irrigation.
- Lettuce plants were irrigated with overhead sprinklers.
- For the sake of our study, a single plot on the lettuce farm was selected for microbiological assessment.



Process flow

After harvesting, the lettuce heads were packed into unlined plastic crates (six heads per crate), stored temporarily in a chilled packhouse and transported in a chilled truck at 5°C to the processing factory about 60 km from the farm. Upon receiving at the factory, the crates were chilled overnight at 5°C in a high humidity refrigerator for processing the following day. After overnight chilling, the external leaves and the lettuce core were removed, and the lettuce leaves were shredded using knives and cutting boards. The leaves were conveyed into an agitated water bath containing 200 ppm chlorine water at pH 4.8, rinsed in another water bath, dried by centrifugation after entering the high care area and packaged in 150 g packs. The processing factory has documented Hazard Analysis and Critical Control Points (HACCP) and is in the process of acquiring HACCP certification.

Sampling and sample preparation

On the farm, irrigation water samples were taken once every two weeks during week 1 (a day after planting), weeks 3, 5, 7 and 10 when the mature lettuce heads were harvested. All samples were taken sequentially by following the same crop through growing, processing, packaging and storage. On the farm, samples were taken once every two weeks on week 1 (a day after planting), weeks 3, 5, 7 and 10 when the mature lettuce heads were harvested. Six lettuce samples and irrigation water samples were taken on the farm. In the processing factory, 6 lettuce samples were taken at each sampling point. Lettuce packs from the processing factory were stored in the laboratory at 5°C for 8 days to simulate retail shelf life. During the storage period, 6 lettuce packs were sampled on each of days 2, 4, 6 and 8 for microbiological analyses. Additionally, three swabs of food contact surfaces were also taken from the farm during harvesting of lettuce and at the processing factory during lettuce processing. Water and lettuce samples were processed as described for irrigation water and lettuce in the previous section (3.2.10).

Microbiological analysis

Total aerobic colony counts (ACC), Intestinal enterococci counts (IE), total coliform counts (TC), *E. coli* counts and *Staphylococcus aureus* counts were determined as described in the experimental procedures for lettuce in the previous section (3.2.10). Typical *E. coli* isolates were further assessed for the presence of Shiga Toxin producing *E. coli* (STEC: stx1, stx2, ehx, eae and O157:H7) and enterohaemorrhagic *E. coli* serotypes (O145, H7, O26, O111 and O103) by real time PCR using gene discs (GeneSystems, France). *Salmonella* spp. and *Listeria* spp. presence/absence were as described in the previous section (3.2.10).

Results

The bacterial incidence in water and on lettuce from planting to end of shelf life were summarised in Table 83.

Water:

Aerobic colony counts (ACC) – the ACC in irrigation water ranged between 3.5 log CFU/mℓ and 5 log CFU/mℓ.

Faecal coliforms – the coliform count ranged between 2.8 log CFU/100 mℓ and 4.1 log CFU/ 100 mℓ

Intestinal enterococci (IE) – the IE counts ranged from zero to1.9 log CFU/mℓ.

Listeria spp. – not detected in irrigation water samples.

Salmonella spp. – present in irrigation water samples.

Lettuce:

Microbiological quality of lettuce and food contact surfaces in the processing plant

The changes in populations of ACC, total coliforms, *S. aureus* and intestinal enterococci (IE) on lettuce sampled at various points along the processing line are illustrated in Figure 53.

Aerobic colony counts (ACC) – ACC for field lettuce samples ranged between 7.2 log CFU/g (week 1) and 6 log CFU/g (week 10). Washing in the packhouse reduced ACC to 3.6 log CFU/g (Fig. 53).

Total coliforms – the maximum count was 6.4 log CFU/g and washing in the packhouse reduced the coliform counts with approximately 3 log CFU/g (Fig. 53).

Intestinal enterococci (IC) – present at low numbers in the field (ranging from zero CFU/g to a maximum of 2.5 log CFU/g). Not present after washing in the packhouse and throughout the shelf life study.

E. coli – present in the field lettuce samples, however no *E. coli* observed at reception in packhouse, after chilling, washing and throughout storage at 5°C for 8 days.

Salmonella spp. – *Salmonella* spp. was detected in lettuce packs on day 8 of storage only.

Listeria spp. – present in field samples, at reception and after overnight chilling, absent after washing and throughout the shelf life study.

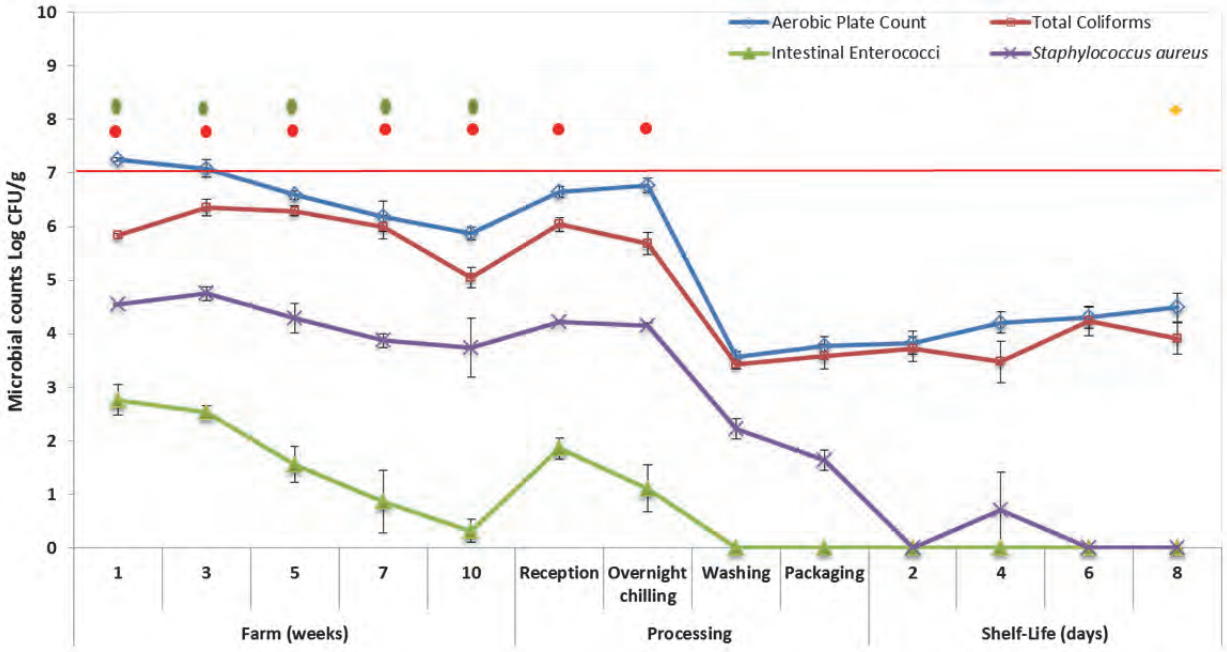


Figure 53: Changes in bacteria concentrations/populations on crisp lettuce from planting to end of shelf life ♦*Salmonella* spp; ● *Listeria* spp; ▮ *Escherichia coli*

Table 83 Summary of bacterial incidence in water and on lettuce from planting to end of shelf life.

		Log$_{10}$ CFU/g or mℓ				% Incidence		
Sampling points	**N**	**Aerobic Plate Count**	**Total Coliforms**	**Intestinal Enterococci**	***S. aureus***	***E. coli***	***Listeria***	***Salmonella***
Irrigation water	5	4.2 (0.54)	3.6 (0.48)	0.9 (0.89)	1.5 (1.11)	60 (3)	ND	40 (2)
Soil	24	6.6 (0.34)	5.7 (0.44)	2.0 (0.93)	4.6 (0.39)	25 (6)	87.5 (21)	ND
Lettuce:								
Farm	30	6.6 (0.64)	5.9 (0.60)	1.6 (1.24)	4.2 (0.76)	10 (3)	63 (19)	ND
Processing factory	30	5.3 (1.44)	4.8 (1.15)	0.7 (0.91)	3.3 (1.21)	ND	10 (3)	ND
Shelf life storage	30	4.1 (0.56)	3.8 (0.71)	0 (0)	0.5 (0.76)	ND	ND	10 (3)

ND: Not Detected

The bacteria isolated from irrigation water, soil and lettuce along the entire supply chain are summarised in Table 84.

Table 84 Bacteria isolated from irrigation water, soil and lettuce along the entire supply chain

Organism type	Isolates	Pathogenic status	Irrigation water	Soil	Lettuce (farm)	Lettuce at receiving	During processing	Shelf-life
Pathogens	*E. coli*							
	E. coli O157:H7[a]	NP						
	Salmonella	NP						
Listeria	*Listeria ivanovii*	P						
	Listeria welshimeri	NP						
	Listeria grayi	NP						
	Listeria innocua	NP						
Intestinal Enterococci	*Enterococcus faecium*	OP						
	Enterococcus faecalis	OP						
	Enterococcus avium	NP						
	Aerococcus viridans	NP						
	Globicatella saguinis	NP						
Coliforms	*Acinetobacter baylyi*	OP						
	Aeromonas caviae	OP						
	Agrobacterium tumefaciens	NP						
	Enterobacter cloacae	OP						
	Enterococcus gallinarum							
	Enterobacter cowanii	U						
	Pantoea agglomerans	P						
	Pseudomonas rhodesia	NP						
	Pseudomonas cichorii	NP						
	Pseudomonas koreensis							
	Pseudomonas oryzihabitants	OP						
	Pseudomonas monteilii	OP						
	Raoultella ornithinolytica	OP						
	Stenotrophomonas sp.							

Pathogen status: P (Pathogenic), NP (Non-pathogenic), OP (Opportunistic pathogen), U (Unknown),

[a]*E. coli* O157:H7 did not have the toxin genes stx1, stx2, eae and ehx characteristic of Shiga Toxin producing *E. coli*. Shaded columns indicate positive samples for the respective bacterial isolates

Table 85 Antibiotic resistance profiles for *Enterococcus faecium* and *Enterococcus faecalis* isolated from lettuce field samples

Antibiotic	*Enterococcus faecalis* (N = 4)	*Enterococcus faecium* (N = 9)
	% Resistance	
Amoxicillin (10 µg)	0 (0)	11 (1)
Ampicillin (10 µg)	0 (0)	0 (0)
Erythromycin (15 µg)	0 (0)	11 (1)
Nitrofurantoin (300 µg)	75 (3)	67 (6)
Tetracycline (30 µg)	25 (1)	22 (2)
Vancomycin (30 µg)	75 (3)	11 (1)
Gentamicin (10 µg)	100 (4)	22 (2)
Ciprofcoxacin (10 µg)	100 (4)	56 (5)

Table 86 Correlation of total coliform counts with the incidence of pathogens on lettuce.

Total coliforms	N	% Positive samples		
		Escherichia coli	*Listeria spp.*	*Salmonella spp.*
<100	0	0	0	0
100-1000	5	0	0	0
1000-10000	19	0	0	5 (1)
>10000	60	5 (3)	37 (22)	3 (2)

Discussion and conclusion

In this study, faecal coliform levels in irrigation water often exceeded the upper limit (≤ log 3 CFU/100 mℓ) suggested for total faecal coliform counts in irrigation water used for irrigation of vegetables eaten uncooked (DWAF, 1996). The use of overhead irrigation on the lettuce production farm contributes significantly to the risk of contamination with human pathogenic bacteria and viruses. Irrigation water from the Crocodile River and Hartbeespoort Dam was susceptible to faecal contamination from informal settlements and wild animals. Proper monitoring of the microbiological quality and partial treatment prior to use could reduce the risk of produce contamination.

Any effective and pragmatic approach to mitigating risks of microbiological contamination in the lettuce supply chain requires the identification and understanding of contamination sources and management strategies to reduce risks. Our study showed that aerobic bacteria, coliforms, intestinal enterococci, *S. aureus, E. coli, Listeria* and *Salmonella* occurred on lettuce. In this study, the increase in aerobic colony counts, total coliform counts and intestinal enterococci on lettuce heads between harvesting and reception at the processing factory suggest the possibility of temperature abuse if harvested lettuce heads were left to stand at ambient temperatures for extended periods prior to chilling. However, further increase after overnight chilling indicates the presence of mesophilic microorganisms that can

multiply under chilling conditions. Washing and rinsing in chlorine water at 200ppm reduced the aerobic colony counts by 3 log CFU/mℓ. Similarly, Ruiz-Cruz *et al*. (2007) demonstrated a 2 to 3 log CFU/g reduction of *L. monocytogenes, Salmonella* and *E. coli* O157:H7 on fresh cut carrots after washing in chlorinated tap water at 200 ppm. Chlorine effects cell death by producing hypochlorous acid and hypochlorite ions in solution which oxidizes bacterial cell membrane components, to cause disruption of cell membrane integrity and release of cell interior (Gómez-López *et al*., 2009). Modified atmosphere packaging further restricted increase in bacterial numbers. On the other hand, restriction of utility microorganisms may have enabled survival and possibly growth of pathogens that occur in low numbers (Farber, 1991). This was evident in Salmonella survival and detection at the end of shelf life storage.

Food safety systems encompass tools that have been developed for the delivery of safe food products along the entire production, manufacturing and distribution chain, provided they are diligently applied in the design and implementation of the supply chain (Gorris, 2005). Global GAP and HACCP implemented on the farm and processing factory respectively, provide a clear guidance to hazard control and ensure due diligence (Gorris, 2005). According to the suggested coliform limit for fresh produce eaten raw (DoH, 2000), the coliform levels were unacceptably high after processing and at the end of shelf life. The South African Guidelines for environmental health officers on the interpretation of microbiological analysis of food has proposed a limit of <200 CFU/g coliform count in raw fruits and vegetables (DoH, 2010). With the high coliform counts it was not surprising that Salmonella was detected in the packaged lettuce during shelf life storage.

According to the suggested coliform limit for fresh produce eaten raw (DoH, 2000), the coliform levels on the lettuce were unacceptably high. A total coliform count of up to 6.4 log CFU/g was observed for the field samples. Although the wash step reduced the ACC, IE, coliform and *S. aureus* counts with approximately 1-3 log CFU/g each, the coliform levels were still high at approximately 3-4 log CFU/g in the lettuce packs. However, although *E. coli* was present in the field lettuce samples, no *E. coli* was observed at reception in the packhouse, after chilling, washing and throughout storage at 5ºC for 8 days. *S. aureus* counts in lettuce packs were < 2 log CFU/g. Salmonella spp. were detected on day 8 in the end of shelf life samples only. *Listeria* spp. were present in field samples, at reception and after overnight chilling, absent after washing and throughout the shelf life study.

3.2.12 NORTH WEST PROVINCE – Potential link between irrigation water quality and the microbiological quality and safety of basil from a farm near Brits

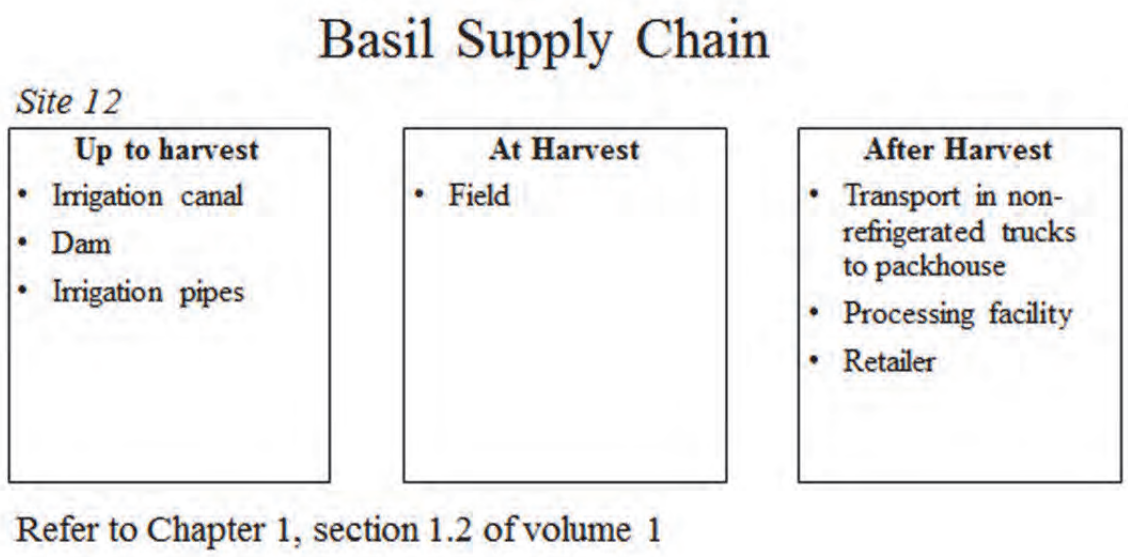
Specific aim

The aim of this study was to evaluate the microbiological quality of the water used for irrigation as well as basil in the field at harvest, after harvest upon arrival at the packhouse and from retailers.

Experimental procedures

Sampling site

Basil was sampled on a commercial farm near Brits (North West Province). Irrigation water is drawn from an irrigation channel, stored in a dam and used for overhead irrigation of the basil.



Process flow

Basil samples are transported to a processing facility near Irene in Pretoria where the herbs are packed and distributed to major retailers in South Africa. Additionally, basil samples were collected from the retailers.

Water and basil sampling and processing

Three different water sites were sampled on each farm, i.e. the water source used, the on-farm storage dam and the outlet of the irrigation pipes. Three 1000 mℓ water samples were extracted from each sampling site during each visit.

Three samples each were obtained from the field, processing facility and from a major retail outlet. The samples were aseptically collected diagonally in the basil field. One basil plant sample consisted of the leaves of three basil plants that were pooled together to add up to a total weight of 40 g. The basil packs are labelled with codes for supplier identification

purposes. Basil packs with the same code were sampled at the retailers. Water and basil processing was as described for parsley in section 3.2.1.

Climatic parameters. Ambient – and water temperatures were recorded throughout the study with an Extech EasyView™ Hygro-Thermometer EA25 (SKC) and an infrared Extech Thermometer (SKC, Johannesburg) respectively.

Microbiological analysis

Total aerobic colony counts, fungi and yeast counts as well as coliform/*E. coli* counts were determined as described previously for water used to irrigate parsley (section 3.2.1).

Molecular methods

Determination of presence of *E. coli* O157:H7 and *S.* Typhimurium using multiplex PCR analysis – DNA was extracted from each of the enriched water samples using the Quick-gDNA miniprep kit (Zymo Research, California, USA). Multiplex PCR analysis was performed using pathogen specific primers and the extracted DNA as template (Cebula *et al*., 1995; Standing *et al*., 2013).

Results

Microbiological analysis

Results of microbiological analysis of the irrigation water are summarised in Table 87.

Aerobic colony counts (ACC) – The ACC counts of irrigation water on the farm ranged between a minimum of 2.8 log CFU/mℓ (channel) and a maximum of 3.28 log CFU/mℓ (storage dam).

Fungi and yeast – The fungi and yeast counts were zero to 0.6 log CFU/mℓ.

Coliforms and E. coli – Coliform counts for the channel water were zero and 3.5 log CFU/100 mℓ water from the storage dam as well as irrigation pipes.

E. coli counts were zero for all water samples.

E. coli O157:H7 – None detected using multiplex PCR analysis.

S. Typhimurium – none detected.

Table 87 Summary of results obtained for microbiological analysis of water used to irrigate basil

Irrigation water sources	Average microbial counts log CFU/g				
	Bacteria	**Fungi**	**Yeast**	**Coliform**	***E. coli***
Channel	4.55 (0.25)	2.5 (0.06)	3.01 (0.4)	4.93 (0.21)	0.00
Storage dam	3.31 (0.56)	2.23 (0)	2.55 (0.44)	3.1 (0.71)	0.00
Overhead sprinklers	4.15 (0.33)	1.98 (0.1)	2.85 (0.39)	3 (0.42)	0.00

Physiological parameters of irrigation water (temperature, relative humidity pH) are summarised in Figure 54.

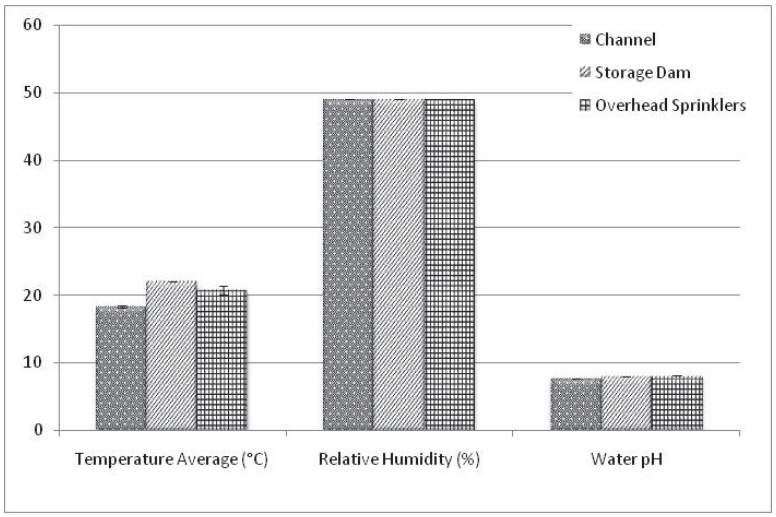


Figure 54: Physiological parameters (water temperature, relative humidity pH) of basil irrigation water samples.

Basil

Aerobic colony counts (ACC) – The maximum ACC count was 4.5 log CFU/g for basil sampled in the field (at harvest) and the minimum count was 3.2 log CFU/g for basil sampled at the packhouse entrance after harvesting (Table 88).

Fungi – The maximum fungal count was 2.5 log CFU/g for the field basil samples and the minimum was 0.7 log CFU/g for basil sampled at the packhouse entrance (Table 88).

Yeast – The maximum yeast count was 3 log CFU/g and the minimum 2.6 log CFU/g for field basil samples (Table 88).

Coliforms and E. coli – Maximum of 4.9 log CFU/g for field basil samples and a minimum of 3 log CFU/g for basil from the retailer. The *E. coli* count for all the basil samples were zero.

E. coli O157:H7 – None detected using multiplex PCR analysis. Two of the basil samples collected at harvest tested positive for the presence of *S.* Typhimurium. Both samples were collected at the point entering the packaging line and were taken at the beginning of the day.

Table 88 Summary of microbiological analysis results obtained for basil.

Produce	Average microbial counts log CFU/g				
	Aerobic bacteria (ACC)	**Fungi**	**Yeast**	**Coliform**	***E. coli***
Field	4.55 (0.25)	2.5 (0.06)	3.01 (0.4)	4.93 (0.21)	0.00 (0)
Packhouse	3.31 (0.56)	2.23 (0)	2.55 (0.44)	3.1 (0.71)	0.00 (0)
Retailer	4.15 (0.33)	1.98 (0.1)	2.85 (0.39)	3 (0.42)	0.00 (0)

Discussion and conclusion

Since the *E. coli* counts of the irrigation water were zero it can be considered suitable for irrigation of any crop, including produce likely to be eaten raw, according to the DWAF guidelines (1996). *E. coli* O157: H7 and *Salmonella* spp. were not detected in any of the irrigation water sources sampled. Other potential pathogens predominantly isolated from the water included *Klebsiella pneumoniae* spp. *pneumoniae* and *Enterobacter aerogenes*. Average pH of irrigation water sources was pH 7 which is acceptable according to the DWAF guidelines. Although the irrigation water quality was acceptable according to the guidelines, the microbiological quality of the basil was not within the required specifications since coliform, yeast and mould counts were found to be higher than that specified by the DoH guidance document for ready to eat fruit and vegetables. Furthermore, *Salmonella* Typhimurium was present in some of the basil samples tested and could pose a health risk, since it is often consumed raw in salads.

3.2.13 WESTERN CAPE PROVINCE – Potential link between irrigation water quality and the microbiological quality and safety of pears from four commercial farms

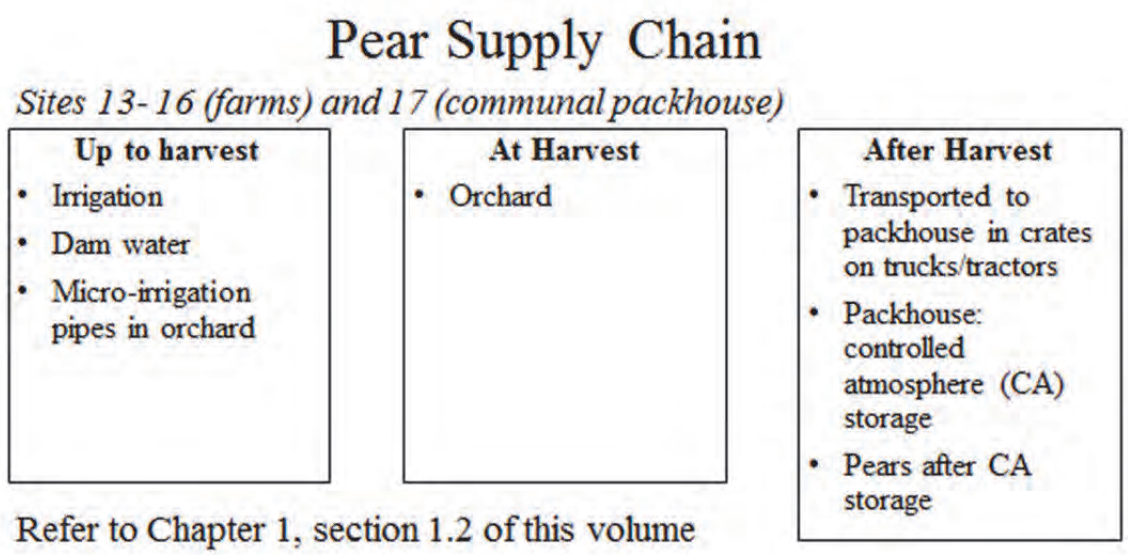
Specific aim:

The aim of this study was to evaluate the microbiological quality of the irrigation water and pears with the ultimate aim to establish a potential link between the irrigation water and the fruit.

Experimental procedures

Experimental sites

- Four commercial pear production farms in the Western Cape
- Pears (*Pyrus communis* cv Packham's Triumph) sent to a communal packhouse from the four farms were sampled after drenching on arrival and again after controlled atmosphere (CA) storage.



Process flow

After harvesting in the orchards, the pears are transported in crates on trucks to a central packhouse. At the packhouse the pears are drenched once with water containing 75 ppm chlorine. After drenching the pears are stored in controlled atmosphere (CA) for 14 weeks, at oxygen levels set at 1.5% (variation 1.2-2.0%), carbon dioxide levels at 1.5% (consistency held with the aid of scrubbers) and temperature set at -0.5°C (with variation at front and back of the room 0.2°C-minus 0.7°C), where after the pears are exported.

Water and pear sample collection and processing

Irrigation water samples were collected from dams and micro irrigation pipes in the orchards on each of the four commercial pear farms in the Western Cape Province as described in section C1. Water used for drenching the pears upon arrival at the communal packhouse was also analysed.

Four replicates (five pears each) were sampled from the orchards of each of the four farms, after drenching at the packhouse as well as after CA storage in the communal packhouse. The pears, picked earlier the same day, were sampled randomly from crates from each of the four farms after drenching at the packhouse. The pear surface temperatures were data logged (Extech Instruments, IR Thermometer).

The respective pear samples were placed into 1 litre Ringer's with 0.02% Tween 80 solution, the displacement volumes determined, and sonicated in a Digital Ultrasonic Cleaner (EUmax®, Labotec, Model no. UD200SH-6L) for 5 min to remove the surface bacteria for enumeration purposes.

Microbiological analysis

Total viable bacterial, coliform/*E. coli* and fungi/yeast counts were determined as described in section 2.3.8 for onion irrigation water analysis.

Each of the filtered water samples were enriched for determining the presence of *E. coli* (including *E. coli* O157:H7) and *Salmonella* by incubation of the 9 mℓ 3M PBW with the filter membranes at 37 °C for 24 hours. Additionally, a millilitre of the 3M PBW broth was transferred into a 9 mℓ tube of 3M *Listeria* selective broth for enrichment purposes and incubated at 37°C for 24 hours.
Subsequently, a loopful of each of the water and pear samples (3M PBW enriched) were streaked onto eosin methylene blue differential medium (EMB) for detection of typical *E. coli* colonies, *Salmonella* brilliance medium for detection of typical *Salmonella* colonies and *Listeria* selective medium for detection of typical *Listeria* colonies.

Identification of presumptive pathogens using Matrix Assisted Laser Desorption Ionisation-Time of Flight (MALDI-TOF)
Single presumptive pathogen colonies isolated from the selective chromogenic media after selective enrichment were identified using Matrix Assisted Laser Ionisation-Time of Flight (MALDI-TOF) as described for the onions in section 2.2.8.

Molecular analysis
Escherichia coli O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. Enterica serovar Typhimurium presence was determined using multiplex PCR analysis as described by Standing *et al*. (2013).

Molecular Detection System (MDS) analysis
The 24h 3M PBW and 3M *Listeria* specific enrichment broths were used to determine the presence/absence of *E. coli*, *Salmonella* spp. and *Listeria* spp. using the respective 3M MDS kits: 3M Molecular Detection Assay *Salmonella* (AOAC RI Certificate 031208, April 2012), 3M Molecular Detection Assay *E. coli* 0157, including H7, (AOAC RI Certificate 071202, July 2012) and 3M Molecular Detection Assay *Listeria* (AOAC RI Certificate 081203, August 2012).

Results
Microbiological analysis of irrigation water
Aerobic colony counts (ACC) – The counts ranged between a minimum of 2.2 CFU/ml and a maximum of 4.09 log CFU/ml for the irrigation dams and micro-irrigation pipes (Table 89).
Coliforms and E. coli – *E. coli* count was zero for farms 1 and 2 for storage dams and in the micro-irrigation pipes. Farms 2 and 3 *E. coli* counts ranged between 1 log CFU/100 mℓ and 2.3 log CFU/100 mℓ (Fig. 55). *E. coli* count for drench water was 0.4 log CFU/100 mℓ (Table 89).
Yeast and fungi – yeasts were counted for irrigation water (Table 89).
E. coli O157 (including H7) – detected in 0/57 water samples using 3M Molecular Detection System (MDS) and multiplex PCR analysis.
Salmonella – detected in 3/57 water samples using 3M Molecular Detection System (MDS).
S. Typhimurium – 2/57 water samples using multiplex PCR analysis.
Listeria spp. – detected in 2/57 water samples using 3M MDS.
L. monocytogenes – 2/57 water samples using multiplex PCR analysis.

Identification of bacteria, fungi and yeasts isolated from water

Drench water: Bacteria isolated included *Bacillus badius* and *Dothideomycetes* spp.

Micro irrigation water: Bacteria included *Chryseobacterium* spp., *Curtobacterium flaccumfaciens, Frigoribacterium* spp. *and Lodobacter* spp.. Fungi included *Cephalo thecasulfurea* and *Paecilomyces lilacinus;* Yeasts included *Ryptococcus laurentii* and *Rhodotorula glutinis.*

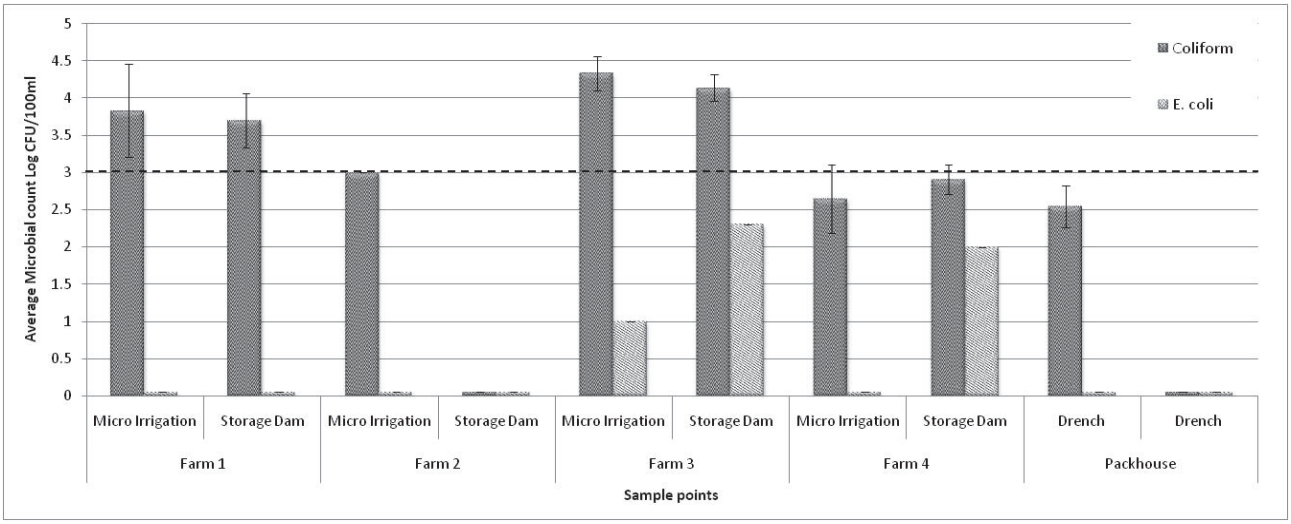


Figure 55: Total coliform and *E. coli* counts measured for irrigation water from four commercial farms in the Western Cape.

Table 89 Summary of microbial counts in irrigation water sources on four commercial pear farms in the Western Cape and drench water at the communal packhouse

Pear farms	Irrigation water source	Microbial counts log CFU/mℓ			Microbial counts log CFU/100mℓ	
		Aerobic bacteria (ACC)	Fungi	Yeast	*Coliforms*	*E. coli*
Farm 1	Storage Dam	2.19 (0.11)	0 (0)	0 (0)	3.83 (0.36)	0 (0)
	Micro Irrigation	3.36 (0.35)	2.12 (0.04)	0 (0)	3.7 (0.61)	0 (0)
Farm 2	Storage Dam	2.5 (0.15)	0 (0)	0 (0)	3 (0)	0 (0)
	Micro Irrigation	2.48 (0.24)	0 (0)	0 (0)	0.05 (0.6)	0 (0)
Farm 3	Storage Dam	3.12 (0.19)	2.02 (0.33)	0.7 (1.21)	4.34 (0.18)	1 (0.28)
	Micro Irrigation	4.09 (0.16)	1.37 (1.22)	1.3 (1.13)	4.14 (0.23)	2.3 (0.6)
Farm 4	Storage Dam	2.77 (0.16)	2.16 (0.12)	0 (0)	2.65 (0.17)	0 (0.17)
	Micro Irrigation	3.26 (0.2)	2.42 (0.15)	0 (0)	2.91 (0.32)	2 (0)
Packhouse	Drench Water	3.02 (0.18)	1.32 (1.14)	0.63 (1.09)	2.54 (0.39)	0 (0.63)

Physico-chemical analysis

The pH of the dam water from the four farms ranged from 6.8 and 7.4. The pH of the water in the micro irrigation pipes varied between 6.8 and 7. Turbidity ranged between < 5-20 for the irrigation water samples (Table 90).

Table 90 pH and turbidity of irrigation water sources from the commercial pear farms and the Drench water at the communal packhouse

Pear farms/ Communal packhouse	Irrigation water source			
	Dam		Micro irrigation	
	pH	Turbidity	pH	Turbidity
Farm 1	7.2	5	7	15
	7.2	5	6.8	15
	7.2	5	6.8	20
Farm 2	6.8	<5	6.8	7.5
	6.8	<5	6.8	7.5
	6.8	<5	6.8	7.5
Farm 3	6.8	10	6.8	<5
	6.8	10	6.8	<5
	6.8	10	6.8	<5
Farm 4	7.4	15	6.8	15
	7.4	10	6.8	20
	7.4	10	6.8	17
Drench water at communal packhouse	8.2	7		
	8.2	7		
	8.2	7		
	7.4	20		
	7.4	20		
	7.4	20		

Microbiological analysis of the pears

Aerobic colony counts (ACC) – The counts ranged between a minimum of 2.2 CFU/cm2 and a maximum of 3.5 log CFU/cm^2 for the orchard, after drenching and after CA storage pear surfaces (Table 91).

Coliforms and E. coli – Coliform counts were low, i.e. a maximum count of 1.9 log CFU/ cm^2 was obtained. *E. coli* counts were zero for the orchard, after drenching and after CA storage pear surfaces (Table 91).

Yeast and fungi – After drenching the combined yeast and mould counts were lower when compared to the orchard samples, the numbers increased again after CA storage. The counts ranged between a minimum of 1.9 log CFU/ cm^2 to a maximum of 5.9 log CFU/ cm^2 (Table 91).

E. coli O157 (including H7) – none detected using 3M MDS and multiplex PCR analysis.

Salmonella spp. – none detected using 3M MDS.

Listeria spp. – detected in two pear samples only out of 36 pear samples analysed.

Table 91 Summary of microbial counts measured for pear surfaces from four commercial farms in the Western Cape

Pear farms	Sample source	Microbial counts log CFU/cm^2				
		Bacteria	**Fungi**	**Yeast**	**Coliforms**	***E. coli***
Farm1	Orchard	2.75 (0.33)	2.19 (0)	1.29 (0)	1.65 (0.55)	0.05 (0)
	After Drench at communal packhouse	2.15 (0.53)	0.45 (0)	0.58 (0)	1.46 (1.23)	0.05 (0)
	After CA	3 (0.22)	1.24 (0)	2.6 (0)	1.7 (0.55)	0 (0)
Farm 2	Orchard	3.2 (0.5)	3.09 (0)	0.56 (0)	1.82 (0.97)	0.05 (0)
	After Drench	2.84 (0.45)	1.05 (0)	2.01 (0)	1.54 (1.13)	0.05 (0)
	After CA	2.56 (0.56)	1.04 (0)	2.32 (0)	1.25 (0.39)	0.05 (0)
Farm 3	Orchard	3.53 (0.3)	2.67 (0)	3.21 (0)	1.92 (0.89)	0.05 (0)
	After Drench	3.32 (0.4)	3.43 (0)	0.05 (0)	0.89 (0.35)	0.05 (0)
	After CA	2.81 (0.82)	1.23 (0)	3.15 (0)	0.27 (0.36)	0.05 (0)
Farm 4	Orchard	3.25 (0.41)	2.35 (0)	3.05 (0)	0.89 (0.26)	0.05 (0)
	After Drench	3.09 (0.48)	3.63 (0)	0.05 (0)	1.09 (0.4)	0.05 (0)
	After CA	2.36 (0.49)	1.39 (0)	2.67 (0)	1.1 (0.15)	0.05 (0)

CA= Controlled atmosphere storage of oxygen levels set at 1.5% (variation 1.2-2.0%), carbon dioxide levels at 1.5%

Discussion and conclusion

The *E. coli* counts in the irrigation water from the four commercial farms ranged from zero to 2.3 log CFU/100 mℓ, which is within the DWAF and WHO irrigation water guidelines for produce not to be eaten raw. *E. coli* O157 (including H7) and *Salmonella* spp. were detected in 2/57 water samples, and detected in 2/57 water samples, and *Listeria* spp.in 6/57 water samples. The pH of the irrigation water samples were within the guidelines set by DWAF's Target Water Quality Range (TWQR) of pH 6-pH 9 (DWAF, 1996).

The coliform counts for the fruit were lower when compared to most of the vegetable products and complied with the DoH guidelines. The *E. coli* count was zero, and no *E. coli* O157 (including H7) or *Salmonella* spp. were detected in any of the pear fruit samples. Although *Listeria* spp. were detected in one enriched pear sample using the 3M MDS, Listeria monocytogenes specifically was not detected in any of the samples after streaking onto chromogenic media and identified using MALDI-TOF analysis. Recently Holfelder *et al.* (2013) reported that chromogenic media gave variable results and should be combined with other identification methods, which is in agreement with our findings.

3.3 SUMMARY OF RESEARCH FINDINGS

GAUTENG PROVINCE – Microbiological quality (including virological analysis) of irrigation water and potential link to the microbiological quality and safety of parsley from a farm in the Elandsdrift area

According to the South African irrigation water quality guidelines the *Escherichia coli* count for safe consumption of crops eaten raw should be ≤1 (CFU/100 mℓ) (DWAF, 1996). The World Health Organization recommended that the faecal coliform limit be relaxed to 1 000 faecal coliforms/100 mℓ for vegetables eaten raw (WHO, 1989).

Water (borehole, Crocodile River, holding dams 1 and 2, sprinklers, centre pivot and nursery) and parsley samples (from the field at harvest, bunches in the packhouse and after packaging) were collected during 2011. The *E. coli* levels in the irrigation water samples were higher than the South African Department of Water Affairs (DWAF) guidelines for irrigation of fresh produce to be eaten raw and could result in the transmission of human pathogens (DWAF, 1996). None of the human pathogenic bacteria (*E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella enterica* subsp. *enterica* serovar Typhimurium) and viruses (Norovirus GI [NoV GI], Norovirus GII [NoV II] and hepatitis A virus [HAV]) tested for, were detected in any of the water samples.

Current microbiological specification guidelines by the Department of Health (DoH) for ready-to-eat fresh fruit and vegetables require coliform counts of less than 200/g, yeast and mould counts of < 100 000 (log 5)/g, *E. coli* 0/g and *Salmonella* at 0/25 g. However, it is important to note that the DoH guidelines provide specifications only for ready-to-eat fresh produce and these values should therefore not be directly used in production or even processing systems.

Parsley samples were collected from two fields, from bunches in the packhouse and after packaging in the packhouse. The yeast and mould counts for parsley along the supply chain (including ready-to-eat packaged parsley) were mostly higher that the DoH specifications of < 4 log CFU/g for ready-to-eat fresh fruit and vegetables, although the human pathogens tested for (*Escherichia coli* O157:H7, *Salmonella* Typhimurium, *L. monocytogenes* and *Staphylococcus aureus*) were not detected in the parsley samples. The presence of Norovirus GII on the parsley samples from the field and in the packhouse is a potential health risk, since parsley is often consumed raw. It has been reported that sixty percent of foodborne disease outbreaks in the United States linked to leafy green vegetable consumption between 1973 and 2006 was caused by Norovirus (http://www.sciencedaily.com/releases/2008/03/080317164356). Since Norovirus GII was not present in the irrigation water, the parsley contamination was probably due to human faecal contamination in the field or the presence of the virus on workers hands in the field and/or packhouse. In WRC report no. 1773/4/12 NoV GII.4, NoV GII.2 were detected in irrigation water and NoVGII.1 was detected on cabbage, NoVGII.9 on tomatoes as well as HAV 1B on tomatoes and lettuce.

GAUTENG PROVINCE – Irrigation water quality and potential impact on the microbiological quality and safety of lettuce from a farm in the Elandsdrift area

River water on the farm is collected in a temporary holding dam which is then pumped and distributed to different areas of the farm through the pump house. The site selected for lettuce sampling received water directly from the pump house and not from a secondary reservoir dam. The water the crops received was therefore freshly pumped from the main holding dam into which the river flowed.

Three different water sources on the farm were tested for quality monitoring purposes: (1) the dam water, (2) the pump house water, and (3) water from the irrigation sprinklers on the plot where lettuce samples were collected. Winter Supreme lettuce (*Lactuca sativa L.* var. *capitata*) were collected every week for 12 weeks including at harvest, in the pack house (after harvest) and at a retail store.

The *E. coli* levels in the irrigation water were higher than the South African Department of Water Affairs (DWAF) guidelines for irrigation of fresh produce to be eaten raw and could result in the transmission of human pathogens (DWAF, 1996). In the current study, despite *E. coli* being detected in 71% of the dam water samples, 79% of the pump house samples and 64% of the irrigation point samples, the pathogen was never detected in lettuce samples and in only 28% of root samples and 14% of soil samples. No *E. coli* O157:H7 was detected either in any of the water sources or the lettuce crops (pre- and postharvest), roots or soil samples tested. Despite contaminated irrigation water being implicated as a critical factor in food safety, the lettuce crops studied in the current study showed no signs of contamination by *E. coli*.

GAUTENG PROVINCE – Virological analysis and indicator organism levels of irrigation water and linked strawberry samples in the Pyramid district, Tshwane Metropolitan area

Irrigation water samples and the associated irrigated strawberries were collected monthly for twelve months (September 2012-August 2013). The *E. coli* levels in the irrigation water were higher than the South African Department of Water Affairs (DWAF) guidelines for irrigation of fresh produce to be eaten raw and could result in the transmission of human pathogens (DWAF, 1996). Multiplex *rt* RT-PCR assays for the detection of NoV GI, NoV GII, HAV, sapovirus (SaV), human astrovirus (AstV), human rotavirus (HRV) and mengovirus (PCR control) were optimised. This made the gathering of new data, to determine the role of irrigation water as a source of contamination of irrigated berry fruits in SA, possible.

The HAV virus was not detected in any of the irrigation water samples. Norovirus GI and NoV GII could be detected in 9.1% (1/11) and 36.4% (4/11) of irrigation water samples with the singleplex *rt* RT-PCR assays, respectively. However, with the multiplex *rt* RT-PCR assays NoV GI and NoV GII were detected in 27.3% (3/11) and 27.3% (3/11) of the irrigation water samples. Human AstV, HRV and SaV were detected in 36.4% (4/11), 18%

(2/11) and 9.1% (1/11) of the irrigation water samples collected. NoV GI, NoV GII and HAV were detected in 4.2% (1/24), 20.8% (5/24) and 4.2% (1/24) of strawberry samples, respectively. Genotypic characterisation of the isolates showed the presence of Human Norovirus GII.7 in irrigation water samples and swine NoV GII.18 in the strawberries. This is the first report of swine NoVs in South Africa, and raises the question as to the possibility of zoonotic NoV infection. Although a definite link between the viruses detected in the irrigation water and those on the strawberries could not be established, a more concerning problem of the possible human-swine NoV variants was brought to light.

GAUTENG PROVINCE and KWAZULU-NATAL PROVINCE – Virological analysis and indicator organism levels in irrigation water and potential impact on the microbiological quality of cabbages from street vendors and two farms (small-scale and commercial)

E. coli was not isolated from the borehole sample from the small-scale farm in Gauteng, but the *E. coli* levels in the Gauteng and KZN river water samples were higher than the South African Department of Water Affairs (DWAF) guidelines for irrigation of fresh produce to be eaten raw, and could result in the transmission of human pathogens (DWAF, 1996). However, the values were within South African guidelines for irrigation of crops that are not eaten raw and that are allowed to dry before harvesting (DWAF, 1996). None of the viruses tested for (NoV GI, NoV GII and HAV) were detected in the river and borehole samples from the small-scale farm in Gauteng. The river water sample from the KZN farm tested positive for HAV and negative for NoV GI and NoV GII. The HAV strain was characterised by neighbour-joining phylogenetic tree analysis and grouped within HAV genotype V. The latter finding suggests the strain to be associated with non-human primates, i.e. simian species.

No viruses (NoV GI, NoV GII, and HAV) were detected in any of the cabbage samples collected from four street vendors in Gauteng, a small-scale farm in Gauteng and a farm in KZN. Since no viruses were detected on produce, a direct link between contaminated irrigation water and associated fresh produce could not be established. WRC report no. 1773/4/12 reported the presence of NoVGII.1 on one cabbage sample irrigated with water from the Berg River in the Western Cape. However, no correlation between the occurrence of viruses in irrigation water samples and linked fresh produce samples was evident.

KWAZULU-NATAL PROVINCE – Irrigation water quality and the microbiological quality of fresh produce (broccoli, bell pepper, cabbage, cauliflower, Crisphead lettuce, jam tomatoes, parsley and spinach) from three farms near Cato Ridge, Camperdown and Richmond respectively

During the twelve month sampling period the *E. coli* counts of river water samples from farm A ranged between 1-1000 CFU/100 mℓ and were higher than the South African Department of Water Affairs (DWAF) guidelines for irrigation of fresh produce to be eaten raw and could potentially result in the transmission of human pathogens (DWAF, 1996). *E. coli* counts of borehole water samples exceeded the 1000 CFU/100 mℓ DWAF and WHO guideline limit

only once, which indicated a potential contamination event of the ground water. *E. coli* counts of the irrigation water sources on farm B (dam and borehole water mixture) as well as from farm C (dam water) were zero. Potential pathogens isolated from the irrigation water included *L. monocytogenes, Salmonella* spp., *Shigella* spp. and *Campylobacter* spp.

During the twelve month sampling period the coliform and *E. coli* counts of fresh produce (broccoli, lettuce, jam tomatoes and spinach, cabbage, cauliflower, parsley and bell peppers) from all three farms were often higher than the DoH guidelines for fresh produce to be eaten raw. *E. coli* was detected less frequently in the lettuce irrigated with the borehole water when compared to lettuce irrigated with the river water, i.e. 3/10 (33.3%) and 6/10 (60%) of the sampling times respectively. Other pathogens isolated from the fresh produce included *L. monocytogenes, Salmonella* spp., *Shigella* spp. and *Campylobacter* spp. It was evident that the fresh produce with the highest microbial contamination was the leafy vegetables, such as lettuce and spinach, throughout the season. Crops that are grown closer to the ground are much more susceptible to contamination since they can come into direct contact with the contaminant through splashed soil or manure during irrigation. Additionally, leafy vegetables have a large leaf surface area, which have been shown to have a high water holding capacity and are therefore at a greater risk of contamination during irrigation.

KWAZULU-NATAL PROVINCE – Irrigation water quality (including agrichemical spray water) and the microbiological quality and safety of leafy vegetables (spinach and lettuce) on a farm near Cato Ridge

E. coli counts of irrigation water and agrichemical spray water sampled from a farm in KZN for a period of three months (August-October 2013) were higher than the DWAF and WHO maximum limit of 1000 *E. coli* CFU/100 mℓ. Typical growth of *L. monocytogenes, Salmonella* spp. and *Shigella* spp. were observed. In this study the ACC and *E. coli* numbers increased in irrigation water mixed with pesticides when compared to the levels in irrigation water alone. This result is in agreement with literature reports, i.e. some pesticides have the ability to promote growth of specific microorganisms when mixed with irrigation water. Presumptive *L. monocytogenes, Salmonella* spp. and *Shigella* spp. were not found in the agrichemical spray water samples.

E. coli counts obtained for spinach and lettuce ranged between 3.3-3.7 log CFU/g and 3.8-4.6 log CFU/g. This is unacceptable according to DoH guidelines fo ready-to-eat fruit and vegetables. The heterotrophic bacterial counts of spinach counts ranged between 4.2 and 4.5 log CFU/g and lettuce between 4.5 and 4.6 log CFU/g. Typical growth was also observed for *Shigella* spp., *Salmonella* spp. and *L. monocytogenes* which could pose a potential health risk since lettuce is consumed raw and spinach is also often consumed raw in salads.

KWAZULU-NATAL PROVINCE – Irrigation water quality (including agrichemical spray water) and the microbiological quality and safety of fresh produce (cabbage and lettuce) from two farms near Camperdown

Irrigation water, agricultural spray water and fresh produce (cabbage and lettuce) samples were collected from two farms in KwaZulu-Natal for a period of three months (July-September 2014). The *E. coli* counts were higher than DWAF and WHO maximum limit of 1000 *E. coli* CFU/100 mℓ for water used to irrigate fresh produce to be eaten raw. *L. monocytogenes* and *Salmonella* were isolated from the irrigation water samples and could therefore pose a potential health risk if transferred onto fresh produce during irrigation. Lettuce generally had the higher microbial load compared to cabbage. Total Heterotrophic bacteria (THB) counts ranged from 3.88-7.21 and 3.08-6.74 log CFU/g for lettuce and cabbage, respectively. *E. coli* counts on cabbage ranged between 0.52-1.12 log CFU/g and on lettuce between zero and 3.2 log CFU/g. There was a significant correlation ($p \leq 0.05$) between the mean counts for irrigation water and vegetables on farm A, and highly significant ($p < 0.01$) for irrigation water and vegetables on farm B. The presence of *L. monocytogenes* and *Salmonella* on some of the lettuce and cabbage samples could pose a potential health risk to the consumer.

The mean turbidity levels in the irrigation water exceeded the international turbidity of 0-1 NTU standard for water (DWAF, 1996). The turbidity of the potable drinking water was within the range of 0-1 NTU. The high turbidity levels of the irrigation water could be the result of soil erosion and runoff. There was a positive correlation between the BOD, temperature and pH, and the turbidity levels on farm A. The values for TDS recorded on both farms were within the recommended range of 1000 mg/l for TDS in drinking water and 40 mg/ml for irrigation water as specified by WHO (WHO, 2012). There was a positive correlation between the TDS and COD levels in the irrigation water from both farms. *E. coli* counts appeared to be influenced by TDS and COD on farm B. Microbial growth could have been supported by the presence of organic matter in the irrigation water.

The BOD levels were lower than the COD levels which is expected since BOD only measures organic material compared to COD which measures both inorganic and organic matter in the water samples. There was a negative correlation between COD and BOD levels on both farms. COD and TDS values were the only two parameters of water that seemed to have influenced the survival of the THB in the irrigation water on farm A. TDS seemed to have also influenced the survival of *E. coli*, *Salmonella* spp. and *L. monocytogenes* in the irrigation water sources on both farms. There was a significant positive correlation between the COD levels and the THB and *E. coli* counts in the water samples. South Africa does not have any guidelines for COD and BOD. However, the COD values recorded exceeded those recommended by WHO of 10 mg/l. This is an indication that the water contains organic pollutants which could help promote the growth of certain microbes in the water. There was a positive correlation between the THB and *E. coli* in the water on farm A while there was a negative correlation between the two in water from farm B. THB counts were also positively correlated wth the *Salmonella* counts.

This study also showed that pesticide solutions used for agricultural spray purposes to control pests and insects can provide a suitable environment for the survival and growth of pathogenic microbes, such as *L. monocytogenes*, *E. coli*, *Salmonella* and *Shigella* spp., whereas others are inhibitory to these microorganisms. Microbe growth was favoured mainly by the pesticides with organophosphates and carbamates as the active ingredients. Organophosphate pesticides contain diazinon and chlorpyrifos. Other active ingredients were abamectin (16-membered macrocyclic lactone derivative), phtalic acid diamide and poly-1-p-methene (insect growth regulator).

LIMPOPO PROVINCE – Potential link between irrigation water quality and the microbiological quality and safety of onions and tomatoes from a commercial farm near Polokwane

The aim of this study was to determine the microbiological quality, prevalence of human pathogenic bacteria and to characterise *E. coli* isolates from irrigation water (river, associated water storage dam, water from overhead irrigation pivot point in the onion field and micro-irrigation pipes in the tomato field) as well as fresh produce (onions and tomatoes) in order to establish a possible link between the water quality and the potential transfer of human pathogens from the irrigation source to the fresh produce.

Water: Although the *E. coli* counts measured for the primary irrigation water source (river) on the farm exceeded the DWAF and WHO guideline limit of 1000 CFU/100 mℓ during the summer months, the *E. coli* counts in the river, dam and irrigation pivot point water during the winter months (during the onion production cycle) were within South African guidelines for irrigation of crops that are not eaten raw and that are allowed to dry before harvesting, i.e. 1-1000 CFU/100 mℓ (DWAF, 1996).

No *E. coli* O157:H7 and *Listeria* spp. were isolated, however *Salmonella* was isolated from 22% of the water samples tested and included *Salmonella enterica* subsp. *enterica* serovar Typhimurium. The MALDI-TOF MS was applied successfully as an alternative method for identification of *E. coli*, *Salmonella* and *L. monocytogenes* as well as other enterobacteriaceae from environmental sources. Noroviruses (NoV GI and NoVGII) as well as the Hepatitis A virus (HAV) were detected in the Sand River, dam and irrigation pivot point water samples. Noroviruses are the most common cause of foodborne illness and outbreaks of NoV gastroenteritis associated with the ingestion of contaminated fresh produce are well documented.

The electrical conductivity (EC) of the water samples ranged from 70.9 mS/m to 115.2 mS/m which can be regarded as good and tolerable respectively according to the DWAF irrigation water guidelines (DWAF, 1996). Nitrogen occurs predominantly as nitrate in irrigation water. The ammonium form is usually a result of contamination with waste water. The highest level of ammonium (25.5 mg/ℓ) was indeed recorded for the water sampled from the dam 3 outflow just below the Polokwane waterworks. The waterworks often overflow after rain and subsequently lead to contamination of the river water downstream which is used for irrigation

purposes on the farm. The pH of all the water samples tested were within the recommended pH range for irrigation water in South Africa, i.e. from pH 6.5 to pH 9.5 (DWAF, 1996). However, this range also promotes growth of enteric pathogens.

Onions: No *E. coli* was counted for any of the onion samples. Additionally, the number of coliform, fungi and yeast counts on the onion surfaces gradually decreased over the sampling period from the 3-4 leaf onion growth stage to the market. At this stage the onion samples complied with DoH general microbiological guidelines for ready-to-eat fresh fruit and vegetables. None of the human pathogenic bacteria tested for were isolated from the onion surfaces, i.e. *E. coli* O157:H7, *Salmonella* Typhimurium and *Listeria monocytogenes*. *E. coli* was however isolated from 10% of the onion samples after selective enrichment, indicating its presence at levels lower than the standard detectable limit for plate counts.

Tomatoes: The microbiological counts for the tomatoes were within the limits of the DoH specifications for ready-to-eat fresh fruit and vegetables. None of the human pathogenic bacteria (*E. coli* O157:H7 and *S.* Typhimurium) tested for were present in the tomatoes. Although water used to irrigate the tomatoes on the farm in Limpopo was contaminated with human enteric viruses including HAV and NoV GI and NoV GII, indicating potential human faecal pollution, none of the viruses were isolated from the tomatoes. In WRC report no. 1773/4/12 NoV GII.4 and NoV GII.2 were detected in irrigation water and NoVGII.9 and HAV 1B on tomatoes from a street vendor in Marabastad.

Linking studies: E. coli was isolated from 78.4% water samples and 8.3% onion samples after selective enrichment and streaking onto appropriate media. The *E. coli* isolates were subjected to antibiotic resistance, virulence gene and Eric-PCR analysis in order to establish whether the strains from the irrigation water sources and onions were similar on a phenotypic and genetic level respectively.

Of the 45 *E. coli* isolates, 15.5% were resistant to one antibiotic and 42.2% were resistant to multiple antibiotics. High resistance to ampicillin and tetracycline was observed for all the irrigation water samples. A high resistance to ampicillin was noted in irrigation water and onion *E. coli* isolates. This is in agreement with results reported for *E. coli* isolates from the Umgeni and Palmiet rivers in KZN in 2009 (Olaniran *et al*., 2009). A comparison of clustering patterns generated with antibiotic resistance profiles of *E. coli* isolated from irrigation water sources (river, dam and irrigation pivot point) and onions clearly showed a link between the irrigation water and fresh produce isolates.

In total 11% of the *E. coli* isolates were positive for at least one virulence gene. The individual virulence genes (*eae, stx1, and stx2*) presence varied between 2.2% to 6.6% of the *E. coli* isolates respectively. Eric PCR analysis clearly grouped some of the river, dam, irrigation pivot point and onion samples at similarity values ranging between 80% and 92% which showed the link between the irrigation water sources and the onion isolates.

Cluster analysis of ERIC-PCR DNA fingerprints of the *E. coli* isolates (100 in total) from several farms, where fruit and vegetables were produced, were done. One *E. coli* isolate from the micro-irrigation pipe water (tomato fields) and one from the washwater (packhouse) formed a separate distinct cluster at a similarity value > 90%. Furthermore, *E. coli* isolates from the borehole, dam and micro-irrigation pipes water formed distinct clusters at similarity values > 80%. These results indicated that the water from the borehole and storage dam was most likely the source of the strain isolated from the micro-irrigation pipes in the fields. *E. coli* was not isolated from the tomato samples after selective enrichment, therefore genetic fingerprinting to determine the link between the irrigation water and tomatoes could not be done.

LIMPOPO PROVINCE – Potential link between irrigation water quality and the microbiological quality and safety of peaches from a commercial peach farm near Mookgophong

The *E. coli* levels in the irrigation water (Sterk River, dam, micro irrigation pipes in the orchard and filters in the irrigation pipes) exceeded the DWAF and WHO guideline limit of 1000 CFU/100 mℓ. Total average bacterial and yeast counts were higher for samples taken from irrigation pipes after filters as well as in micro-irrigation pipes in the orchard when compared to river and dam water counts. This could indicate an accumulation of microorganisms in the filters as opposed to improving the water quality by installing filters in the irrigation pipes in the peach orchard due to the filters not being replaced regularly.

None of the human pathogenic bacteria tested for (*E. coli* O157 (including H7), *Salmonella* spp. and *Listeria* spp.) were isolated from the water samples. The microbiological quality of the peaches were mostly acceptable according to DoH guidelines. *E. coli* was detected on orchard peaches only, *E. coli* O157 (including H7), *Salmonella* spp. and *L. monocytogenes* were not detected in any of the fruit samples.
Phenotypic (antibiotic resistance) characterisation of *E. coli* isolates from the peaches (two in total), river (one isolate) and micro irrigation pipes (one isolate) showed that the strains from the irrigation water and peaches were sensitive to all the antibiotics (14 in total) tested. Genotypic characterisation showed that both the *stx1* and *stx2* virulence genes were present in the *E. coli* isolate from the river. Linking studies based on genotypic and phenotypic characterisation could not be done, due to the limited number of *E. coli* isolates obtained from the irrigation water and peaches.

NORTH WEST PROVINCE – Potential link between the microbiological quality of irrigation water and the microbiological quality and safety of lettuce from a farm near Skeerpoort

Irrigation water and Crisphead lettuce samples were collected monthly from a field irrigated with water from the Skeerpoort River for 10 months from January to October 2011. During the ten month sampling period the faecal coliform counts exceeded the DWAF guideline limit of *E. coli* (1000 CFU/100 mℓ) only once. The pH ranged from 7.9 to 9.2 in the Skeerpoort

River and was acceptable according to the South African national guidelines (DWAF, 1996). The river water temperature ranged from 10.7 to 26.0˚C with the lowest and highest noted in May and January respectively.

Faecal coliform (FC) counts of up to 3.5 log CFU/g lettuce were observed which is higher than the DoH specifications to be used as guidelines for the microbiological quality of fresh produce. Over the ten month sampling period FC were present during 5 of the 10 (monthly) sampling intervals. *E. coli* was present during 3 of the 10 sampling intervals. *S. aureus* counts were present during 9 of the 10 sampling intervals. *E. coli*, *Bacillus* spp. and *Enterobacter* spp. were the most prevalent bacterial genera isolated from lettuce. *E. coli* was the most prevalent species on lettuce in winter. The sporadic counts noted for bacterial indicators on lettuce coupled with diversity of isolated bacterial species on lettuce suggested contamination from various environmental sources such as contaminated irrigation water, soil, animals as well as human handlers.

The potential link between the Skeerpoort River and lettuce irrigated with this water was evaluated by determining the antibiotic resistance profiles of *E. coli* isolated from these two sources. Resistance of isolates to at least one antibiotic was 83.3% in the Skeerpoort River (n=12). From lettuce (n=10), 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 the two samples was to cephalothin and ampicillin. High resistance (33.3%) was noted to multiple (more than 2) antibiotics in the Skeerpoort River. Most isolates from the same source showed close relatedness. Forty percent of *E. coli* isolates from irrigated lettuce showed close phenotypic relatedness to isolates from the Skeerpoort River. There were 33% of isolates from irrigation water having 100% genetic similarity to 60% of isolates from lettuce.

Genetic relatedness of *E. coli* isolates from irrigation water and lettuce was determined using GTG PCR DNA fingerprinting. There were 33% of isolates from irrigation water having 100% genetic similarity to 60% of isolates from lettuce. Results from this study show that *E. coli* irrigation water harbours antibiotic resistant *E. coli* which can be transferred onto irrigated produce thereby compromising food safety and public health. The result for lettuce specifically was in agreement with WRC Report Number 1773/1-4/12) linking studies which showed at least 30 percent correlation between irrigation water and pathogen presence on the product.

NORTH WEST PROVINCE – Potential link between irrigation water quality (irrigation channel fed by Crocodile River and Hartbeespoort Dam) and microbiological quality and safety of Crisphead lettuce from harvesting to end of shelf life in the Brits area

In this study the faecal coliform counts in the water ranged between 2.8 log CFU/mℓ and 4.1 log CFU/100 mℓ and mostly exceeded the upper limit (≤ 3 lg CFU/100 ml) of the DWAF guidelines for irrigation water for fresh produce to be eaten raw. *Listeria* spp. was not detected in irrigation water samples. *Salmonella* spp. was isolated from the irrigation water

and could pose a health risk when used to irrigate lettuce to be eaten raw. *Listeria* spp. was not detected in irrigation water samples. *Salmonella* spp. were however isolated from the irrigation water and could pose a health risk when used to irrigate lettuce to be eaten raw. High incidence of *Listeria* spp. was observed in field lettuce and the corresponding soil samples. *E. coli* incidence was however low in field lettuce (10%) and soil (25%) but high in irrigation water (60%).

According to the suggested coliform limit for fresh produce eaten raw (DoH, 2000), the coliform levels on the lettuce were unacceptably high. A total coliform count of up to 6.4 log CFU/g was observed for the field samples. Although the wash step significantly reduced the aerobic colony (ACC), Intestinal enterococci (IE), coliform and *S. aureus* counts with approximately 1-3 log CFU/g each, the coliform levels were still high at approximately 3-4 log CFU/g in the lettuce packs. Most of the coliforms isolated from lettuce surfaces were also present in the irrigation water and soil. *Enterobacter cowanii, Pantoea agglomerans, Pseudomonas Rhodesia* and *Pseudomonas koreensis* were the predominant coliforms isolated from lettuce at reception and during processing.

However, although *E. coli* was present in the field lettuce samples, no *E. coli* was isolated from lettuce samples at reception in packhouse, after chilling, washing and throughout storage at 5°C for eight days. *E. coli* and *Listeria* were only detected when coliform numbers exceeded 10000 CFU/g. *Salmonella* was detected when total coliforms exceeded 1000 CFU/g. Although *E. coli* O157:H7 was isolated from water and lettuce samples, they did not carry the toxin genes *stx1, stx2, ehx* and *eae*.

The predominant IE species were *E. faecium* and *E. avium*. These were present in both the irrigation water and on lettuce throughout the cultivation period. Other species isolated included *E. faecalis, Aeromonas viridians* and *Globicatella saguinis*. Further characterization of antibiotic resistance of *E. faecium* and *E. faecalis* isolated from field samples indicated high resistance of *E. faecalis* to vancomycin, gentamicin, ciprofloxacin and nitrofurantoin compared to *E. faecium*. Unlike *E. faecium, E. faecalis* was not detected in irrigation water.

S. aureus counts in lettuce packs were < 2 log CFU/g. *Salmonella* spp. were detected on day 8 in the end of shelf life samples only. *Listeria* spp. were present in field samples, at reception and after overnight chilling, but absent after washing and throughout the shelf life study.

NORTH WEST PROVINCE – Potential link between irrigation water quality and the microbiological quality and safety of basil from a farm near Brits

Since the *E. coli* counts of the irrigation water were zero, it could be regarded as suitable for irrigation of any crops, including crops likely to be eaten raw, according to the DWAF guidelines (1996). Additionally *E. coli* O157: H7 and *Salmonella* spp. were not detected in any of the irrigation water sources. Other potential pathogens predominantly isolated from the water included *Klebsiella pneumoniae* spp. *pneumoniae* and *Enterobacter aerogenes*. The average pH of irrigation water sources was pH 7 which is acceptable according to DWAF guidelines.

Although the irrigation water quality was acceptable according to DWAF guidelines, the microbiological quality of ready-to-eat packaged basil was unacceptable since coliform, yeast and mould counts for basil (field, packhorse and retailer) were higher that the DoH specifications. Furthermore, *Salmonella* Typhimurium was present in some of the packhouse basil samples and could pose a potential health risk, since basil is mostly consumed raw.

WESTERN CAPE PROVINCE – Potential link between irrigation water quality and the microbiological quality and safety of pears from four commercial farms

The *E. coli* counts in the irrigation water from water storage dams and micro irrigation pipes in the orchards from the four commercial farms ranged from zero to 2.3 log CFU/100 mℓ and were higher than the South African Department of Water Affairs (DWAF) guidelines for irrigation of fresh produce to be eaten raw and could result in the transmission of human pathogens (DWAF, 1996). *E. coli* O157 (including H7) was not detected in any of the water samples. However, *S.* Typhimurium and *L. monoctogenes* were detected in 3.5% of the samples. The pH of the irrigation water samples were within the guidelines set by DWAF's Target Water Quality Range (TWQR) of pH 6-pH 9 (DWAF, 1996).

The *E. coli* counts were zero and *E. coli* O157 (including H7) and *Salmonella* spp. were not detected in any of the pear fruit samples which complied with DoH guidelines. *Listeria* spp. was detected in one pear sample using the 3 M Molecular Detection System. The fungi/yeast, coliform and *E. coli* counts for the pears were generally lower when compared to most of the other vegetable crops, especially the leafy green vegetables analysed in this study, were acceptable according to the DoH guidelines for fresh produce to be eaten raw. It should be kept in mind that pears irrigated with micro-irrigation pipes are mostly not in direct contact with the irrigation water. Low hanging fruit in the orchards might however be contaminated with human pathogenic bacteria present in the irrigation water.

The *E. coli* isolates from the water samples from the four commercial pear farms were mostly sensitive to the antibiotics (14 in total) tested. However, resistance to single antibiotics were detected for some *E. coli* isolates and included either Aztreonam (ATM), Ceftazimide (CAZ) (which both belong to the Penicillin class of antibiotics) or Chloramphenicol (C) (which belongs to the Phenicol antibiotic class). Only one isolate was resistant to two antibiotics which included ATM and CAZ. The single pear *E. coli* isolate from pears in the orchard of one of the farms was sensitive to all the antibiotics tested. None of the virulence genes were detected in any of the *E. coli* isolates (18 in total) from the irrigation water on the four commercial pear farms in the single pear *E. coli* isolate. Cluster analysis of ERIC-PCR DNA fingerprints showed that two *E. coli* isolates from the dam water and two isolates from the micro-irrigation pipes in the orchard, on one of the farms, formed a distinct cluster at an 80% similarity value. This result confirmed that the water from the storage dam was the source of the strain isolated from the micro-irrigation pipes.

General conclusions

Several tested sites showed contamination at point source. Data from irrigation water studies confirmed the results reported previously, namely that the *E. coli* levels in the irrigation water sources often exceeded the maximum of ≥1000 CFU/100 mℓ stipulated by the WHO and DWAF. Current microbiological specification guidelines for ready-to-eat fresh fruit and vegetables require coliform counts of less than 200/g, yeast and mould counts of < 100 000 (log 4)/g, *E. coli* 0/g and *Salmonella at* 0/25 g (DoH, 2000). The microorganism levels on crop surfaces depended on the type of crop and were generally much lower further down the supply chain. *Staphylococcus aureus*, *Listeria* spp., *Salmonella* spp., commensal *E. coli* and diarrhoeagenic *E. coli*, as well as viruses (NoV GI, NoV GII, and HAV) were isolated from fresh produce. Waterborne human pathogens were detected at certain stages along the supply chain, but not from the beginning to the end of the chain. Environmental conditions such as exposure to high temperatures, UV and minimal processing steps have been reported to contribute to a reduction in pathogen numbers. Other potential pathogenic microorganisms isolated included *Enterococcus faecalis*, *Enterobacter cloacae*, *E. kobei* and *Klebsiella pneumoniae*.

Our studies show the accuracy of results can be correlated with sample size and test methods used. Sample size must be adequate to obtain a statistically validated result as far as pathogen presence/absence is concerned. Incidence studies provide a snapshot of contamination at a specific study site and on a specific crop at a particular time during the year. Therefore, caution should be exercised when drawing conclusions from any result based on biased sample size.

A multi-method approach is advisable to determine pathogen prevalence to enhance food safety risk management systems in the supply chain, since selective chromogenic media traditionally used for identification of presumptive human pathogenic bacteria gave unreliable results. It is recommended to initially use a molecular detection method such as 3M Molecular Detection System (MDS) or Multiplex Polymerase Chain Reaction (PCR), followed by isolation of a single presumptive colony from samples that tested positive. The 3M MDS methodology is based on isothermal DNA amplification and bioluminescence detection, which has been reported to increase the sensitivity of pathogen detection. The multiplex method uses pathogen specific primer sets to detect multiple pathogens in a single sample. In this study the MDS and multiplex PCR method results correlated with each other. The identities of single colonies should be confirmed using PCR and Matrix Assisted Ionisation Time Of Flight (MALDI-TOF). MALDI-TOF analysis was initially used for identification of clinically important pathogens. However, a number of publications have successfully applied the technology for fast reliable identification of microorganisms isolated from the environment including water and fresh produce. Results obtained in this study confirmed these reports in the literature and emphasized the importance of food safety management systems to limit foodborne pathogen presence in the fresh produce supply chain.

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CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

4.1 CONCLUSION

WRC Report 1773/1-4/12 "*Quantitative Investigation into the Link between Irrigation Water Quality and Food Safety*" focused primarily on the river water quality and its impact on fresh produce. In this follow-up study "*Investigation into the link between water quality and microbiological safety of selected fruit and vegetables from farming to processing stages*" (WRC project K5/1875/4), irrigation river and dam water quality was analysed in the context of assessing any link with the fresh produce up to at-, and after-harvest, as well as at the point of sale. The most important findings of this study are summarised in this Chapter. Microbial analysis demonstrated that the levels of *Escherichia coli* in the irrigation water (five rivers, ten dams) at the fourteen study sites exceeded the DWAF (1996) irrigation water guidelines of ≤1000 CFU/100 mℓ for crops that are eaten raw. However, irrigation canal water used at two of the sites tested complied with the DWAF (1996) guidelines for irrigation water for crops to be eaten raw. Although the *E. coli* counts of the primary irrigation sources (rivers and dams) were unacceptably high, a reduction in numbers was observed for dam water and for water collected from the micro-irrigation pipes, drip-irrigation and overhead irrigation pivots.

Salmonella was isolated from up to 22% of irrigation water samples tested from a selected site. However, no *E. coli* O157:H7 or *Listeria* spp. were detected in these samples (Du Plessis *et al*., 2015). The presence of *E. coli*, hepatitis A virus (HAV), sapovirus (SaV) and norovirus (NoV) suggested that the contamination was most likely of human origin, possibly from the upstream sewage works. These pathogens pose a potential health risk to the end-user of such contaminated fresh produce.

The mean turbidity levels in irrigation water from a site in KwaZulu-Natal often exceeded the international turbidity standard of 0-1 NTU's for water (DWAF, 1996). The high turbidity levels of the irrigation water could be the result of soil erosion and runoff. In this study it was found that there was a positive correlation between the biological oxygen demand (BOD), temperature and pH, and the turbidity levels in the water. The values for total dissolved solids (TDS) recorded were within the recommended range of 1000 mg/l for drinking water and 40 mg/ml for irrigation water as specified by the World Health Organisation (WHO, 1996). There was a positive correlation between the TDS and the chemical oxygen demand (COD) levels in the irrigation water from two farms in KwaZulu-Natal. It was concluded that the *E. coli* counts could have been influenced by TDS and COD in irrigation water from one of the farms.

The BOD levels of the irrigation water sources from selected farms were lower than the COD levels, which can be expected, since the former only measures organic material compared to COD which measures both inorganic and organic matter in the water samples. There was a negative correlation between COD and BOD levels. The COD and TDS values were the only

two parameters of water that influenced the survival of total heterotrophic bacteria (THB), *E. coli*, *Salmonella* spp. and *L. monocytogenes* in the irrigation water sources on both farms. *There was thus a significant positive correlation between the COD levels, the THB, E. coli and Salmonella counts in the water samples.* It is important to note that South Africa does not have any guidelines for COD and BOD. However, the COD values recorded exceeded those recommended by the World Health Organization (WHO) of 10 mg/l.

The effect of irrigation methods (drip, sprinkler and flood) on the surface contamination of fresh produce was found to be crop dependant. The findings of this study demonstrated that irrigation methods that minimize direct contact of the contaminated water with the edible part of the plant lowered the risk of contamination to the fresh produce.

Hazard characterization was also performed on several fresh produce supply chains, including a variety of fruit (tomatoes, peaches, pears, strawberries), vegetables (lettuce, spinach, cabbage, onions and broccoli), herbs (parsley, basil) as well as processed produce (fruit salad and frozen vegetables). The current national guideline for fresh produce provides guidance values only for the for ready-to-eat stage of fresh produce in the chain (DoH, 2000). The microbial levels on crop surfaces were generally found to be higher in the field compared to the product at the end of the supply chain. *Escherichia coli* and diarrhoeagenic *E. coli*, as well as viruses (NoV GI, NoV GII, and HAV) were isolated from fresh produce at certain stages along the supply chain**,** *but not throughout the chain.* The fresh produce with the highest microbial contamination was leafy vegetables (lettuce and spinach). Interestingly, swine NoV GII.18 was detected in the strawberries. This was the first report of swine NoVs in South Africa, which raised the question of the possibility of zoonotic NoV infection.

Phenotypic (antimicrobial) and genotypic (virulence gene prevalence, DNA fingerprinting) analysis of *E. coli* isolates clearly showed a link between the irrigation water source/s (river, dam, irrigation pivot point) and fresh produce isolates at two of the selected sites. *Generally the percentage of pathogens present on vegetable products was higher when compared to fruit samples*. This is an important finding, since it confirms previous reports that fruit in general is by its very nature a lower-risk product than vegetables. This is mainly due to production practices where the crop is grown in closer proximity to the ground and the potential to be in direct contact with irrigation water for extended periods of time is thus greater. However, some fruit types cannot be grouped under this broad category as was found for strawberries in this and the previous WRC project (WRC Report Number 1773/1-4/12). Strawberries are grown close to the ground, have a corrugated surface and were found to harbour several viruses.

Furthermore, internalisation studies confirmed the ability of pathogens to be taken up, survive, internalise, multiply in plant tissue and move up to the leaves. Once internalised, the pathogens are protected against the effect of minimal processing steps such as chlorine washing or surface disinfection employed to promote food safety in the supply chain. Environmental conditions such as exposure to high temperatures, UV and minimal processing steps (chlorine washing, hydrogen peroxide treatment, cooking, blanching, microwaving and freezing) contributed to a reduction in microorganism numbers including human pathogenic

bacteria. *It was found in our study that the effectivity of minimal processing steps to reduce microbiological surface contamination levels is crop dependant* due to the surface characteristics, i.e. smooth versus hairy and/or rough surfaces. Agricultural chemicals can be used in the supply chain to reduce foodborne-pathogen-associated hazards, but the fact that some of the chemicals can support pathogen growth should be taken into consideration in a risk assessment strategy for each crop.

The general aim of this study, to investigate the links between water quality and microbiological safety of fresh minimally processed and frozen fruit and vegetables, was achieved. However, it can be concluded that the accuracy of results can be directly correlated with sample size and test method used. Sample size must be adequate to obtain a statistically viable result as far as pathogen presence/absence is concerned. Incidence studies further provide a snapshot of contamination potential at a specific study site and on a specific crop at a particular time during the year. Therefore, caution should be exercised when drawing conclusions from any result based on biased sampling and sample size. A similar new focus should be on isolation strategies and confirmation of identities of the isolated organisms. Having a sound assessment strategy and using different test methods could provide a more accurate reflection of the real situation. Therefore, testing is as good as the methods used. Careful interpretation of results is thus key to an effective food safety management system. It is also important to highlight that increased testing will not solve the problem and only increase the cost. *The bottom line is prevention of contamination of irrigation water sources.*

There is no doubt that the quality of South Africa's river water has gone beyond a critical point and more effective management strategies of all the waterways and in particular agricultural water, is key to securing safe food for the future. Food safety assurance should therefore be seen in this context. However, despite the fact that the South African rivers are polluted and currently exceed the requirements for agricultural irrigation water, no consistent direct link could be made to contaminated produce up to the point of sale. *Fresh produce in South Africa remains a low risk food product* given its nutritional quality and health benefits and current volumes consumed. Preventative measures such as good agricultural practices, good hygiene practices, good sanitation practices and food safety management systems can ensure safe food. Keeping in mind that foodborne disease outbreaks in totality, given the volume of produce consumed, is still relatively low. However, one foodborne disease outbreak is one too many and all possible precautions should be taken and preventative strategies employed from the water source to the point of sale.

A general statement can be made: ***"Fresh produce can be considered safe and the national population has never before had a bigger variety of consistent supplies of safe, nutritious fresh produce".*** However, foodborne pathogens were on occasion isolated from water sources, the environment and even produce, but could not be linked to the final product at the point of sale. Addressing food insecurity in the context of food safety assurance is thus relevant in a country where fresh produce remains more available to higher income groups. Keeping in mind that the emphasis on the "right-to-food" and more specifically "safe food" is a basic human right, the national Department of Water and Environmental Affairs,

Department of Agriculture Forestry and Fisheries and the Department of Health (DWA, DAFF and DoH) should provide clear guidance, policies and regulatory frameworks based on sound scientific evidence to ensure safe food for all. Effective enforcement, sampling and self-regulation are the basic pillars of an effective food landscape and for sustainable economic growth and prosperity.

4.2 RECOMMENDATIONS FOR FURTHER RESEARCH

Fix the source of the problem: Preventative strategies

The source of the problem contributing to the high level of microbial pollution in South African rivers, should be addressed in a holistic manner. Results obtained during the five-year investigation confirmed previous findings that water sources are seriously compromised and have the potential to negatively affect the health and well-being of the country's people. Some of the most important reasons for the contamination include the presence of informal settlements with inadequate sanitation and lack of access to potable water, inadequate municipal waste water management services, increased pressure on waterworks due to lack of capacity, accelerated urbanisation rate and increased population. Continued regular monitoring of irrigation water sources must be implemented with a view to developing a database of information and an early warning system. Currently, a national water monitoring system exists using accredited laboratories. However, this information is not centrally managed, and neither are forecasts made nor early-warning systems deployed.

Need for updating applicable regulations

The need for national water quality monitoring linked to an early warning system is paramount for national water security. This study clearly showed the variation in data obtained from different regions and for different crops. It is clear from the findings that a database for the presence of foodborne pathogens should be established, maintained and relevant information provided to consumers, retailers, producers and exporters. Effective management systems should also be developed, based on adequate statistically-designed sampling plans, sampling-methods and -intervals to provide a clear national picture of the status of microbial safety of fresh produce.

Revision of current microbiological guidelines for irrigation water and fresh produce based on scientific data (including actual natural microbial levels and pathogen presence/absence) are needed. The World Health Organization (WHO) recommended that the faecal coliform limit be relaxed to 1000 CFU/100 mℓ for irrigating agricultural crops and for vegetables eaten raw (WHO, 1989), which is a more realistic count taking results of the current study into consideration. In addition, more realistic, fit-for-purpose guidelines for irrigation water should be developed based on sound scientific evidence. Commercial farmers seldom pump water directly from the rivers onto the crops but usually have a holding dam and then irrigate from this source. In the current study it was found that the microbial load differed significantly between the water used for irrigation versus river water. However, this constitutes a different problem with small-scale farmers often being totally reliant on river water. Alternative practices should therefore be developed for such situations.

Sampling methods

It is clear from this and the previous study that the test method will influence the sensitivity and accuracy of results and that old standard microbiological methods are no longer fit-for-purpose. These methods have been referenced in most of the regulations dealing with food safety and should be revised as a matter of urgency. A whole new range of technologies are currently being developed that will change the diagnostic platforms for microorganisms. These include, amongst others, next-generation sequencing, real-time PCR and the various genomics platforms, which will increase accuracy and provide more timely results that will allow for effective product recall. Rapid, sensitive and accurate test methods will become the norm as source tracking and traceability in the supply chain improve.

Best farming and manufacturing practices

Good agricultural practices and production and processing methods are essential for all to employ, including small-scale farmers. More effective management of water quality will be essential in all farming systems. Risk mitigation strategies such as systematic and regular pipe maintenance should therefore be adopted. Strategies to decontaminate agricultural water is also important and should be investigated and scientific findings adopted by industry. On-farm storage of water in underground reservoirs could be an option for growers to reduce the level of microbial pathogens. Direct irrigation of crops from contaminated rivers should be prevented and risk-mitigation strategies developed to circumvent this.

Risk assessment approach.

In this study, hazard characterization was carried out on several crops and supply chains. Commodity-specific supply-chain management systems and guidelines, based on scientific data (including actual natural microbial populations and pathogen presence/absence) should therefore be developed. Since information on the distribution of the five major types of diarrheagenic *E. coli* in irrigation water and on fresh produce in South Africa is limited, future work should focus on determining the prevalence of virulence genes and serotypes associated with *E. coli* isolated from food and water sources.

Although viruses of human origin are considered to be a greater risk to human health, the emergence of potentially zoonotic viruses, e.g. hepatitis E virus, recombinant NoV and human-animal rotaviruses (RV), in food and water sources need to be monitored and managed at a national level. Therefore, public health surveillance of food and water sources for these viruses is essential for food safety assurance.

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ANNEXURES

Annexure I Post-graduate training of students and human capacity building (thesis and dissertations awarded and in progress)

Students who have completed their post-graduate training as part of the project K5/1875/4.

Institution	Student	Gender	Race	Degree awarded
University of Pretoria, Department of Microbiology and Plant Pathology	V van der Merwe	Female	White	BSc Hons (Microbiology)
University of Pretoria, Department of Microbiology and Plant Pathology	T-A Standing	Female	White	BSc Honours (Microbiology)
University of Pretoria, Department of Microbiology and Plant Pathology	A Gerber	Female	White	BSc Honours (Microbiology)
University of Pretoria, Department of Microbiology and Plant Pathology	K Snyders	Female	White	BSc Hons (Microbiology)
University of Pretoria, Department of Microbiology and Plant Pathology	M Harris	Female	White	BSc Hons (Microbiology)
University of Pretoria, Department of Microbiology and Plant Pathology	S Collignon	Female	White	PhD (Microbiology)
University of Pretoria, Department of Food Science	M Crous	Female	White	MSc (Food Science)
University of Pretoria, Department of Food Science	G Duhain	Female	White	MSc (Food Science)

Institution	Student	Gender	Race	Degree awarded
University of Pretoria, Department of Food Science	M Muller	Female	White	MSc (Food Science)
University of Pretoria, Department of Food Science	M Ajuika	Male	Black	MSC (Food Science)
University of Pretoria, Department of Food Science	S Dube	Female	Black	MSC (Food Science)
University of Pretoria, Department of Medical Virology	P Matsapola	Male	Black	BSc (Hons) (Medical Virology)
University of Pretoria, Department of Medical Virology	R Saïd	Female	Black	MSc (Medical Virology)
University of Pretoria, Department of Medical Virology	V Ruhanya	Male	Black	MSc (Medical Virology)
University of Pretoria, Department of Medical Virology	G De Ridder	Male	White	MSc (Medical Virology)
University of KwaZulu-Natal, Department Microbiology	C Sam	Female	Indian	BSc Honours (Microbiology)
University of KwaZulu-Natal, Department Microbiology	P Padayachee	Female	Indian	BSc Honours (Microbiology)
University of KwaZulu-Natal, Department Microbiology	F Moosa	Female	Indian	BSc Honours (Microbiology)
University of KwaZulu-Natal, Department Microbiology	L Mvelase	Male	Black	BSc Honours (Microbiology)
University of KwaZulu-Natal, Department Microbiology	TC Mandulane	Female	Black	BSc Honours (Microbiology)

Institution	Student	Gender	Race	Degree awarded
University of KwaZulu-Natal, Department Microbiology	L Mkize	Female	Black	BSc Honours (Microbiology)
University of KwaZulu-Natal, Department Microbiology	A Naidoo	Female	Indian	MSc (Microbiology)

Students on course for graduation

Institution	Student	Gender	Race	Degree
University of Pretoria, Department Microbiology and Plant Pathology	V van der Merwe	Female	White	PhD (Microbiology)
University of Pretoria, Department Microbiology and Plant Pathology	Francois J Duvenage	Male	White	MSc (Microbiology)
University of KwaZulu-Natal, Department Microbiology	Sarasha Govender	Female	Indian	MSc (Microbiology)

Annexure II Journal publications, conference papers, workshops and technology transfer activities

Publications

1. Aijuka M, Charimba George, Hugo CJ and Buys EM (2015) Characterization of bacterial pathogens in rural and urban irrigation water. Journal of Water and Health **13**, 103-117.
2. Du Plessis EM, Duvenage F and Korsten L (2015) Determining the Potential Link between Irrigation Water Quality and the Microbiological Quality of Onions by Phenotypic and Genotypic Characterization of *Escherichia coli* Isolates. Journal of Food Protection **78**, 643-651 (doi:10.4315/0362-028X).

3. Murray TY, Taylor MB (2015) Quantification and molecular characterisation of human sapoviruses in water sources impacted by highly-polluted discharged wastewater in South Africa. Journal of Water and Health (doi:10.2166/wh.2015.255).

4. Rachida S, Matsapola PN., Wolfaardt M, Taylor MB (2015) Genetic characterisation of a novel hepatitis A virus strain in irrigation water in South Africa. Journal of Medical Virology. Accepted for publication after revision.

5. Saïd R, Wolfaardt M, Taylor MB (2014) Molecular characterisation of hepatitis A virus strains from water sources in South Africa. Water Science and Technology. Water Science Technology **69**, 923-233 (doi: 10.2166/wst.2013.799).
6. Standing T-A, Du Plessis EM, Duvenage S and Korsten L (2013) Internalisation potential of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. Enterica serovar Typhimurium and *Staphylococcus aureus* in lettuce seedlings and mature plants. Journal of Water and Health **11**, 210-223.

7. Cook N, de Ridder GA, d'Agostino M, Taylor MB (2013) Internal amplification controls in real-time polymerase chain reaction-based methods for pathogen detection. In: Rodríguez-Lázaro D, editor. Real-time PCR in Food Science. Current Technology and Applications. Norfolk, UK: Caister Academic Press; 2013. p. 35-42.

8. Duhain GLMC, Minnaar A, Buys EM (2012) Effect of Chlorine, Blanching, Freezing, and Microwave Heating on Cryptosporidium parvum Viability Inoculated on Green Peppers Journal of Food Protection 75, 936-941 (doi:10.4315/0362-028X.JFP-11-367).

9. Taylor MB (2012) Tracing the sources of outbreaks of food and waterborne viral disease, and outbreak investigation using molecular method. In N. Cook (ed), Viruses in food and water: risks, surveillance and control. Woodhead Publishing Limited; Cambridge.

Conferences

National conferences

1. De Ridder GA, Taylor MB, Cook N (2011*)* Amplification controls for the real-time PCR analysis of noroviruses in water and food: which is best [Poster]. 2nd Regional Conference of the Southern African Young Water Professionals 3-5 July 2011: CSIR International Convention Centre, Pretoria, South Africa.

2. Saïd R, Wolfaardt M, Taylor MB (2012) Molecular characterisation of hepatitis A strains from water sources in South Africa [Presentation]. Fountain of Knowledge UP Water Institute Members' Forum Conference 9 November 2012 Law Building, Hatfield Campus, University of Pretoria.

3. Saïd R (2013) Molecular characterisation of Hepatitis A virus strains from watersources in South Africa [Presentation]. 3rd Regional Conference of the Southern Africa Young Water Professionals, 16-18 July 2013, Conservatorium, University of Stellenbosch, South Africa.

International conferences

1. Korsten L, Buys E, Taylor M, Pillay B and du Plessis EM (2013) Dealing with Microbial Hazards Transmitted to Fresh Produce via water: The South African Perspective. International Association for Food Protection (IAFP) Conference, Charlotte Convention Centre, USA, 31 July 2013 [Presentation].

2. Korsten L (2012) Microbial hazards in irrigation waters potential pathogen transmission to fresh produce. ILSI Europe Seminar on Assessing Safety of Water Used in the Production of Fresh Produce. World Water Week, Stockholm, Sweden, August 26-31, 2012 [Presentation].

3. Aijuka M. and E.M. Buys (2012) Is Irrigation Water a Source of Foodborne Pathogens on Irrigated Produce? Food Micro 2012, Istanbul, September 2012 [Poster].

4. Aijuka M. and E.M. Buys (2013) Irrigation Water as a Source of Antibiotic Resistant and Virulent *E. coli* on Lettuce. International Association for Food Protection (IAFP) Conference, Charlotte Convention Centre, USA, 28-31 July 2013 [Poster].

Workshops and technology transfer

1. WRC project K5/1875/4 Workshop at Department Microbiology, KZN University 5-11 July 2009.
2. WRC project K5/1875/4 Workshop at Department Microbiology and Plant Pathology, New Agricultural Building, Hatfield Campus, UP 14 to 18 September 2009.
3. WRC project K5/1875/4 Workshop at Department Microbiology and Plant pathology, New Agricultural Building, Hatfield Campus, UP 30 July-1 August 2012.

4. WRC project K5/1875/4 Workshop at Department Microbiology and Plant Pathology, UP on new microbiology methods and WRC student project progress presentations at Plant Sciences Building, Hatfield Campus, UP 13-15th May 2013.
5. Workshop on Food Safety, Post-harvest Technology and Packaging for the Future, Plant Sciences Building, Hatfield Campus, UP Workshop 20-21 August 2013.

Workshop presentations and posters

Presentations

2012:

1. Ajuka M (2012) Ecology of bacterial pathogens in irrigation water WRC projectK5/1875/4 Workshop 30 July-1 August 2012 Agricultural Building, Hatfield Campus.

2. Duvenage FJ and du Plessis EM (2012) Microbiological quality of irrigation water and onions WRC project K5/1875/4 Workshop 30 July-1 August 2012 Agricultural Building, Hatfield Campus.

3. Govender S (2012) Development and evaluation of multiplex qPCR methods for the detection of foodborne pathogens in fresh produce and irrigation water. WRC project K5/1875/4 Workshop 30 July-1 August 2012 Agricultural Building, Hatfield Campus.

4. Ndindwa N (2012) An investigation into the virological quality of irrigation water and irrigated fresh produce. WRC project K5/1875/4 Workshop 30 July-1 August 2012 Agricultural Building, Hatfield Campus.

5. Ndindwa N (2012) An investigation into the virological quality of irrigation water and irrigated fresh produce. Research meeting, Department of Medical Virology, University of Pretoria, Pretoria 20 July 2012.

6. Said R (2012) Molecular characterisation of hepatitis a virus strains from clinical and environmental sources in South Africa. Research meeting, Department of Medical Virology, University of Pretoria, Pretoria 20 July 2012.

7. Said R, Wolfaardt M, Taylor MB (2012) Molecular characterisation of Hepatitis A virus strains from water sources in South Africa Faculty Day, Faculty of Health Sciences, University of Pretoria 28-29 August 2012: HW Snyman Building North, Pretoria.

8. Simhadri D (2012) Impact of pesticides and environmental parameters on microbial growth on fresh produce. WRC project K5/1875/4 Workshop 30 July-1 August 2012 Agricultural Building, Hatfield Campus.

9. Van der Merwe V (2012) Biofilm formation and water quality in pre-harvest environments. WRC project K5/1875/4 Workshop 30 July-1 August 2012 Agricultural Building, Hatfield Campus.

10. Van Dyk B (2012) Microbial safety of commercially produced tomatoes in South Africa. WRC project K5/1875/4 Workshop 30 July-1 August 2012 Agricultural Building, Hatfield Campus.

2013:

1. Ajuka M (2013) Antibiotic resistance in *E. coli* as a means of assessing bacterial contamination on irrigated lettuce WRC project K5/1875/4 Workshop 13-15 May 2013, Plant Sciences Building, Hatfield Campus, UP.

2. Du Plessis, EM and L Korsten. Water Research Commission Funded Projects @ Department Microbiology and Plant Pathology (2013) UP celebrating the university of Pretoria's water-related knowledge A research awareness event presented by the University of Pretoria's Water Institute28 and 29 October 2013 Sanlam Auditorium (Hatfield Campus), University of Pretoria.

3. Duvenage F (2013) Microbiological survey of pears, pre-harvest up to post-harvest. WRC project K5/1875/4 Workshop 13-15 May 2013 Plant Sciences Building, Hatfield Campus, UP.

4. Harris M (2013) A microbiological survey of ready-to-eat fruit salad before and after packaging and the fate and survival of *Listeria innocua* in ready-to-eat fruit salad. WRC project K5/1875/4 Workshop 13-15 May 2013, Plant Sciences Building, Hatfield Campus, UP.

5. Korsten L, Buys E, Taylor M, Pillay B and du Plessis EM (2013) Potential link between irrigation water quality and fresh produce safety. Workshop 20-21 August 2013: Food Safety, Post-harvest Technology and Packaging for the Future, Plant Sciences Building, Hatfield Campus, UP.

6. Matsapola P (2013) Molecular investigation of selected enteric viruses on cabbages & associated irrigation water in Gauteng & Kwazulu-Natal. WRC project K5/1875/4 Workshop 13-15 May 2013, Plant Sciences Building Hatfield Campus, UP.

7. Matsapola P, Said R and Taylor MB (2013) Molecular investigation of selected enteric viruses on cabbages & associated irrigation water in Gauteng & Kwazulu-Natal. Faculty of Health Sciences Research Day 20-21 August 2013 HW Snyman North, UP.

8. Muller M (2013) The occurrence of bacterial hazards and succession of *Enterobacteriaceae* spp. on fresh-cut fruit during minimal processing WRC project K5/1875/4 Workshop 13-15 May 2013, Plant Sciences Building Hatfield Campus, UP.

9. Said R (2013) Molecular characterisation of Hepatitis A virus strains from clinical and environmental sources in South Africa WRC project K5/1875/4 Workshop 13-15 May 2013, Plant Sciences Building Hatfield Campus, UP.

10. Mundulane C T (2013) Impact of pesticides and irrigation water on the microbial quality of fresh produce WRC project K5/1875/4 Workshop 13-15 May 2013, Plant Sciences Building Hatfield Campus, UP.

11. Van Wyk R (2013) The impact of irrigation water quality on fresh and minimally processed fruit and vegetables. WRC project K5/1875/4 Workshop 13-15 May 2013, Plant Sciences Building Hatfield Campus, UP.

12. Venter F (2013) Implications of environmental *Escherichia coli* in agriculture. Workshop 20-21 August 2013: Food Safety, Post-harvest Technology and Packaging for the Future, Plant Sciences Building, Hatfield Campus, UP.

Posters

2012:

1. De Ridder GA, Taylor MB (2012) Strawberries: Healthy friend of foe? Faculty Day, Faculty of Health Sciences, University of Pretoria 28-29 August 2012: HW Snyman Building North, Pretoria.

2. Ndindwa N, Said R, Taylor MB (2012) An investigation into the virological quality of irrigation water and associated tomatoes. Faculty Day, Faculty of Health Sciences, University of Pretoria 28-29 August 2012: HW Snyman Building North, Pretoria.

3. Ruhanya V, Olorunju S, Taylor MB (2012) Efficiency of glass wool adsorption-elution technique for the recovery of selected enteric viruses from raw and treated water. Faculty Day, Faculty of Health Sciences, University of Pretoria 28-29 August 2012: HW Snyman Building North, Pretoria.

Thesis and Dissertations
Honours studies completed:

BSc Honours (Microbiology) Department of Microbiology and Plant Pathology, University of Pretoria:

1. Van der Merwe V (2009) Characterisation of biofilms in PVC irrigation systems when using different water sources.
2. Standing T-A (2009) Internalisation potential of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Staphylococcus aureus* in lettuce seedlings and mature plant.

3. Gerber A (2010) Detection of foodborne pathogens and quantification of microflora on various fresh produce obtained from retailer.

4. Snyders K (2011) The impact of microbiologically contaminated irrigation water on Parsley safety and quality.

5. Harris M (2013) A microbiological survey of ready-to-eat fruit salad before and after packaging and the fate and survival of Listeria innocua in ready-to-eat fruit salad.

BSc Honours (Microbiology) Department of Microbiology, University of KwaZulu-Natal:

6. Sam C (2009) Effect of different irrigation methods on surface contamination of fresh produce.

7. Padayachee P (2009) Microbiological Quality of Frozen Vegetables.

8. Moosa F (2009) Implications of viable but non-culturable (VBNC) microorganisms in irrigation water on the safety of fruits and vegetables.

9. Mvelase L (2011) Agro-chemical spray water as a potential source of contamination of fruits and vegetables.

10. Mandulane TC (2013) Impact of pesticides and irrigation water on the microbial quality of fresh produce.
11. Mkize L (2014) Assessment of the impact of irrigation water and pesticides on the microbial contamination of crops.

BSc Honours Department of Medical Virology, UP:

12. Matsapola P (2013) Molecular investigation of selected enteric viruses on cabbages & associated irrigation water in Gauteng & Kwazulu-Natal.

Masters studies completed:

1. **Mignon Crous (MSc Food Science) "Effect of irrigation intervals and processing on the survival of *Listeria monocytogenes* on spray irrigated broccoli"**

Abstract: The first aim of this study was to determine the effect of irrigation intervals on the survival of L. monocytogenes on spray irrigated broccoli under field trial conditions, and subsequent survival of the pathogen on broccoli during postharvest processing procedures. The non-pathogenic *Listeria innocua* was used as surrogate organism to *Listeria monocytogenes*.

Broccoli in the field was treated with irrigation water inoculated with *L. innocua*, during intervals over a period of five weeks and the growth and survival of the organism was monitored weekly. *L. innocua* numbers remained similar over intervals that received consecutive inoculations and *L. innocua* numbers decreased by at least 2.3 log CFU/g after inoculation ceased, which showed an inoculation effect and that time had an influence on organism survival. Cessation of irrigation before harvest was found to effectively reduce pathogen contamination levels on the crop, whilst repeated irrigation with contaminated water contributes to maintenance of *L. innocua* as well as elevated total microbial counts on the

broccoli. A lack of correlation between the *L. innocua* counts and the recorded environmental temperatures in the field, including temperature and relative humidity, suggested that survival is not solely dependent on and influenced by, nor can it be predicted by these parameters. It was found that the presence of high levels of contamination (with, in this case *L. innocua*) in irrigation water used for vegetable crops, can be associated with an increased microbial population on the crop surface.

Secondly, the effect of processing on organism survival post-harvest was assessed. Washing with water caused a 1 log reduction of *L. innocua*, whilst washing with 200 ppm chlorinated water facilitated a further 1 log reduction. Cooking reduced *L. innocua* numbers on broccoli by an average of 1.1 log units and aerobic plate counts by between 1 and 2 log units. A combined treatment of washing with chlorine, storage in MAP (5% CO2, 5% O2) for two days at 4°C and final microwave heating resulted in the lowest pathogen numbers, causing a 5.13 log CFU/g reduction. Therefore, even though chlorine is effective in reducing L. innocua during minimal processing, it does not suffice alone to eliminate pathogens (with *L. innocua* being representative of *L. monocytogenes*) from vegetables, just as MAP storage is only effective as part of a hurdle procedure. Cooking is essential in destroying *L. innocua* present on broccoli and to ensure vegetables that are safe for consumption in terms of pathogenic exposure.

With this knowledge on the behaviour of *L. monocytogenes* on broccoli, the risk associated with the application of contaminated irrigation water to fresh produce can be better understood and the hazard managed.

2. **Géraldine Duhain (MScFood Science) "Occurrence of *Cryptosporidium* spp. in South African irrigation waters and survival of *Cryptosporidium parvum* during vegetable processing"**

Abstract: Surface waters used for irrigation purposes in South Africa have been found to be of poor microbiological quality and to be contaminated with human pathogens. These pathogens can be transferred from contaminated water onto fresh produce and potentially cause human infections. Cryptosporidium and Giardia are waterborne parasitic protozoa that have been found in surface waters worldwide. They can cause morbidity in infected individuals and be lethal when infecting people with compromised immunity. This study was divided into two phases.

The first phase was a field survey aimed at determining the incidence of human pathogens Cryptosporidium, Giardia and *Salmonella* spp. in rivers used for irrigation purposes in South Africa as well as on vegetables irrigated with these rivers. The rivers selected where from three different provinces of South Africa. The relationship between faecal indicators and the presence of Cryptosporidium oocysts and Giardia cysts was also investigated. Out of the 30 water samples analysed, 43% were positive for Cryptosporidium oocysts, 23% tested positive for Giardia cysts and 27% were positive for *Salmonella* spp. However, no Cryptosporidium oocysts or Giardia cysts were found on the vegetables analysed. No significant differences in the prevalence of Cryptosporidium and Giardia and indicator parameters were observed

between the three rivers. Using a logistic regression model, no significant correlations were observed between the incidence of faecal indicators and the presence of Cryptosporidium oocysts and Giardia cysts.

In second phase of the study, the individual and combined effects of chlorine, blanching, blast freezing and microwave heating on *Cryptosporidium parvum* oocysts inoculated on green pepper were investigated. The viability of the oocysts after treatments was determined using the vital dye propidium iodide. Stained oocysts were counted with a flow cytometer. Chlorine treatments did not significantly affect the viability of the oocysts. Blast freezing affected 20% of the oocysts. Both microwave heating and blanching affected 93% of oocysts. Combined treatments with chlorine and blast freezing did not affect the viability of the oocysts significantly compared to the control. Combined treatment with chlorine and microwaves was significantly more effective than microwave heating alone and affected 98.1% of the oocysts. The data indicate that *C. parvum* oocysts are sensitive to heat and, to some extent, to freezing temperature but are resistant to chlorine.

The results of the survey show the presence of Cryptosporidium and Giardia in irrigation waters and thus a possible health risk associated with the consumption of raw vegetables as those can become contaminated via the irrigation water. The results of the challenge test indicate that *C. parvum* oocysts on vegetables are inactivated by blanching and microwave heating but survive blast freezing and exposure to chlorine. Boiling and microwave heating of vegetables should be sufficient to kill *C. parvum*. On the other hand, ready-to-eat vegetables could be at risk of carrying live C. parvum oocysts as the use of chlorine in washing bath is not be expected to inactivate *C. parvum* oocysts present on vegetables.

3. **Matthew Aijuka (2013) (MSc Food Science) "Bacteriological quality of South African irrigation water and its role as a source of contamination on irrigated lettuce"**

Abstract: 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 (74.7 mm) than the Loskop canal (0.1 mm). 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 CFU/ml, were noted for ACC, ASF, AnSF, *S. aureus* and IE at both irrigation sites. Higher mean ACC of 5.9 log CFU/g and *S. aureus* counts of 3.0 log CFU/g were noted on lettuce. Although low mean counts of FC (1.3 log CFU/100 ml) 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 in spite 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.

Close relatedness was noted between isolates from the Loskop 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.

4. **Margot Muller (2014) (MSC Food Science) "Prevalance of microbial hazards and succession of Enterobacteriaceae on fresh cut fruit during minimal processing"**

Abstract: In this study, the microbial quality of minimally processed mangos and sweet melons were investigated during minimal processing and storage. In addition, the succession of Enterobacteriaceae on minimally processed sweet melons was studied. Whole fruit, cut fruit as well as packaged fruit at the beginning and end of shelf life were sampled and analysed. The samples were analysed for the total aerobic plate count, Enterobacteriaceae, yeast and moulds, *Echerichia coli*, *E. coli* O157:H7, *Salmonella*, *Listeria monocytogenes* and *Staphylococcus aureus*. The strains of similar Enterobacteriaceae species were characterized by MALDI-TOF MS. *Enterobacter cloacae* species were characterized by PCR fingerprinting using the $(GTG)_5$ microsatellite and MALDI-TOF MS. The total average plate counts for the mangos and melons were was $>10^6$CFU/g and $>10^7$CFU/g respectively. With regards to the pathogens, S. aureus was present on the packaged mango and packaged melon samples at the beginning and end of shelf life. *E. coli* was identified as a dominant Enterobacteriaceae specie on the packaged melons at the end of shelf life, but was not detected during any other preceding processing stage. No *L. monocytogenes* or *Salmonella* isolates were detected. The most predominant specie identified during minimal processing was found to be E. cloacae, which comprised 37% of all identified Enterobacteriaceae isolates. Cluster analysis of a PCR fingerprint and MALDI-TOF MS profiles of several strains of this specie obtained during various stages of processing showed similar grouping of the strains. The analysis showed that there is intragenetic variation amongst strains coming from different processing stages. This may suggest that there may be selection or adaption of some strains of *Enterobacter cloacae* with certain genotypic or phenotypic characteristics due to the changing conditions at the various minimal processing stages. This study shows that minimally processed mango and melon is contaminated with bacterial pathogens which may pose a risk to food safety and public health amongst individuals that consume these food products. It also shows that there may be a possible potential for the use of *E. cloaca*e as a biocontrol agent on fresh-cut melons/fruit? In addition, it shows the potential of MALDI-TOF MS profiles in the fingerprinting of isolates from minimally processed fruits.

Further investigation is required to determine what the possible bacterial contamination sources are and if the critical control points in the minimal processing production chain are in place. Since there are limited methods available for prolonging the shelf life of minimally processed fruit, further investigation for the use of *E. cloacae* as biocontrol agent would be beneficial. The research could be valuable information to the minimal fruit processing industry as it can improve food safety and lower the health risk to the consumer.

5. **Siphiwe Dube 2014 (MSc Food Science) "Effect of accelerated storage temperatures on the shelf life limiting factors of apple juice concentrate"**

Abstract: Apple juice concentrate amongst other uses is used to formulate concentrated and single strength fruit juice blends. An apple juice concentrate whose quality has deteriorated during storage poses problems for the processing industries as it limits its use in the formulation of certain products. A dark coloured apple juice concentrate cannot be used to formulate light coloured beverages while an apple concentrate contaminated with microbes can only be incorporated into products with a pasteurisation treatment. It is important to evaluate the deterioration in the quality of apple juice concentrate stored at normal and at accelerated temperatures.

The physicochemical parameters and microbial counts were monitored in apple juice concentrate stored at 10ºC (normal temperature) and at 25ºC and 35ºC (accelerated temperatures) over 12 weeks after which modelling of the experimental data was done. The Biolog system and $(GTG)_5$ PCR fingerprinting were used to identify isolated yeasts.

There was a significant change ($p \leq 0.05$) in L*, b*, a*, hue, chroma, total colour difference (TCD), non-enzymatic browning index (A_{420nm}) and 5-hydroxymethylfurfural concentration with storage time and temperature. The Q_{10} values for L*, b*, non-enzymatic browning index (A_{420nm}) and 5-hydroxymethylfurfural were 1.53, 1.20, 1.81 and 5.25, respectively and activation energies were 8.17, 7.70 and 14.8 kcal mol^{-1} for L*, b* and non-enzymatic browning index (A_{420nm}), respectively.

Microbial growth was not observed in apple juice concentrate stored at 10ºC while yeast and mould growth was observed after 5 weeks of storage at 25ºC and 35ºC. After fitting the yeast growth data into the Baranyi and Roberts model, no significant difference was observed in the lag phase for yeast growth at 25ºC and 35ºC while there was a significant difference $p \leq 0.05$ in the maximum specific growth rate. The theoretical minimum temperature of growth extrapolated from the Ratkowsky model was about 20ºC. The activation energy and Q_{10} values for the maximum specific growth rate were 39.95 kcal mol^{-1} and 4.47, respectively. The isolated yeasts were identified as *Kluyveromyces delphensis, Saccharomyces dairensis, Zygosaccharomyces bailii, Rhodotorula glutinis* and *Metchnikowia reukaufii* and strain variability was observed for the species *K. delphensis* and *S. dairensis.* The higher Q_{10} and activation energy values for the maximum specific growth rate of yeasts in comparison with the Maillard browning parameters (L*, b* and A_{420nm}) signify that loss in quality due to yeast growth is more rapid compared to Maillard browning.

The results of this study show that under refrigerated storage temperatures deterioration in the quality of apple juice concentrate is due to Maillard browning while at accelerated storage temperatures quality loss is due to both Maillard browning and microbial growth of yeasts. At accelerated storage temperatures, darkening of the apple juice concentrate starts immediately and continues throughout the storage period. However growth of spoilage yeasts is only detected after 5 weeks of storage and proceeds rapidly thereafter resulting in spoilage.

6. **Vurayai Ruhanya (2012) (MSc Medical Virology) "Efficiency of glass wool adsorption-elution technique for the recovery of enteric viruses from water"**

Abstract: One of the major obstacles to human health relates to unsafe water and poor sanitation. Faecal contamination of source and drinking water introduces enteric pathogens which result in disease outbreaks. Therefore monitoring the occurrence of human pathogens in source water and drinking water is necessary in order to limit the prevalence of environmentally transmitted infectious diseases. Knowledge of pathogen loads in source waters provides the basis for establishing treatment requirements and health standards stipulated by water regulatory authorities and assists in determining the efficacy of water treatment plants. Water quality monitoring and public health assurance is performed routinely by enumerating faecal indicator bacteria. Studies have demonstrated that there is no relationship between current bacterial indicator detection and the presence of enteric pathogenic viruses in treated and source water. There is therefore a need to monitor the levels of pathogenic enteric viruses in surface waters, irrigation water, sewage effluent as well as treated drinking water for public health safety and quality assessment. However due to the low concentration of viruses in water matrices and presence of inhibitors, efficient concentration methods from large quantities of water are essential.

The analysis of water for enteric viruses is a two stage process: the first step is to apply efficient viral recovery and concentration procedures from large volumes (10-1000 ℓ) of water followed by viral detection. Glass wool adsorption-elution is a cost-effective and practical viral recovery method for use in resource-limiting settings. The main objective of this study was to determine the efficiency of the glass wool adsorption-elution method for the recovery of viruses of different genera from large water samples (10 ℓ) of different quality by a step-by-step evaluation of its performance using seeding experiments. Standard curves were prepared using quantitative reverse transcription-polymerase chain reactions (RT-PCR)(for RNA viruses) and PCR (for DNA viruses). The efficiency of recovery (EOR) of glass wool between tap water and turbid surface water was compared for six enteric viruses by examining the recovery and loss of viruses at each stage of the process. The generalised linear statistical model was applied to compare the EOR of each virus in each water type and results clearly indicated that the EOR varied for each virus type and was higher for tap water than for turbid surface water for each virus. There was extensive loss of virus in the flow through and this was also higher for the turbid water than the tap water. In this study it was also demonstrated that mengovirus behaved similarly to the pathogenic enteric viruses and was therefore a suitable process control to monitor viral recovery and nucleic acid extraction when recovering and detecting enteric viruses from environmental matrices using glass wool adsorption method. It was also demonstrated that EOR of glass wool for turbid surface water was underestimated as the poor sample quality affected the quantitative molecular detection assays. Adenovirus was shown to be a suitable indicator for virus contamination of water. Modification of the glass wool column preparation did not result in significant difference in EOR but an increase in the amount of glass wool used resulted in reduction in EOR. There were no significant differences between the two polyethylene glycol/sodium chloride (PEG_{6000}/NaCl and PEG_{8000}/NaCl) precipitation methods applied to the secondary

concentration of the viruses, but it should be noted that the former has the disadvantage of overnight incubation. The EOR of glass wool was shown to be influenced by pH of the sample. The optimal sample pH for the recovery of hepatitis A virus in turbid surface water was pH 6.0. The study provides valuable new data on the EOR of enteric viruses using the glass-wool adsorption-elution technique where virus quantities could be traced from seeding to detection by molecular-based methods.

7. **Gabriël A De Ridder (2012) (MSc Medical Virology) "The development and application of multiplex real-time reverse transcription polymerase chain reaction assays for the detection of enteric viruses on berry fruits and in water samples"**

Abstract: The transmission of enteric viruses by food, food products and water remains a well-recognised, largely underestimated widespread public health problem. Outbreaks of gastroenteritis and hepatitis A due to the consumption of contaminated berry fruits have become a growing phenomenon worldwide. ontamination of fresh produce and other minimally processed foods can be attributed to pre- and post-harvest irrigation and washing water and food handlers. The prevention of such outbreaks relies on the optimisation of adequate methods for the recovery and detection of enteric viruses from food matrices and irrigation water.

The aim of this study was to develop and apply optimised multiplex real-time reverse transcriptase-polymerase chain reaction (*rt* RT-PCR) assays for the detection of selected enteric viruses on berry fruits and in paired associated irrigation waters. In this study quality control measures were implemented by the development and optimisation of an internal amplification control (IAC) for norovirus (NoV) GII to monitor for the success of the amplification process. Mengovirus was used as a process control to validate the recovery and nucleic acid extraction of selected enteric viruses from strawberries and in associated irrigation waters. Three multiplex *rt* RT-PCR assays for the detection of NoV GI, NoV GII, sapovirus, hepatitis A virus, human astrovirus, human rotavirus and mengovirus were optimised with the IAC. Blackberries and strawberries were used to assess the efficiency of three nucleic acid extraction kits with the most efficient used in further investigations. Three elution buffers based on protein concentration, pH, Tris and elution period were assessed for the recovery of the viruses from the berry fruits. The pH more so than the protein concentration proved to be more effective in the recovery of the selected enteric viruses from the strawberries with no analytical significant differences noted for the two 3% glycine-beef extract (GBE) buffers assessed, irrespective of the parameters considered. During the period September 2010 to August 2011, strawberries and associated irrigation water were collected from which NoV GII, NoV GI and HAV could be recovered using a 3% tris-GBE pH 9.5 elution buffer and a glass-wool absorption elution method, respectively, and detected using optimised singleplex *rt* RT-PCR assays. The irrigation water samples together with eight surface and three groundwater samples collected from the Limpopo area was retested using the optimised multiplex *rt* RT-PCR assays. The multiplex *rt* RT-PCR assays proved to be more efficient in the detection of NoVs than the commercial environmental *rt* RT-PCR assays

with lower detection efficiencies noted for HAV. Commercially obtained strawberries were dipped in polluted surface water, the viruses recovered from both and detected using the optimised multiplex *rt* RT-PCR assays resulted in the detection of similar viruses on both the strawberries and polluted irrigation water. Norovirus GII.7 and swine NoV GII.18 were identified on the strawberries and in the associated irrigation water, respectively. This is the first report of swine NoVs in South Africa, and begs the question as to the possibility of zoonotic NoV infection. This link between the viruses detected on the surface of the strawberries and in the irrigation water could not be confirmed by typing data. From this study, a functional AC was developed and used in the development and optimisation of three multiplex *rt* RT-PCR assays which made the gathering of new data of the role of irrigation water as a source of contamination of irrigated berry fruits in SA possible.

8. **Rachida Said (2013) (MSc Medical Virology) "Molecular characterisation of hepatitis A virus strains from clinical and environmental sources in South Africa"**

Abstract: Hepatitis A virus (HAV) is the most common cause of acute viral hepatitis worldwide. Hepatitis A virus is classified in the genus *Hepatovirus* of the family *Picornaviridae*. The virion has a 7.5 kb positive-sense single-stranded RNA genome and only one serotype of the virus has been identified so far. There are six genotypes of HAV distinguished by nucleotide sequence analysis of the VP1 region. Genotypes I, II and III, each with their subdivisions A and B, infect humans. Genotypes IV, V and IV infect non-human primates. These genotypes have a distinct geographical distribution. In 1997, it was established that genotype IA and IB prevail in South Africa (SA) with IB being predominant. However, no recent data have been published on the types of HAV circulating in SA even though the virus was detected in surface water sources and on fresh produce at the point of retail. There is an effective vaccine for the prevention of hepatitis A, but recent publications have reported the possible emergence of antigenic escape mutants of HAV. The aim of the study was therefore to establish which genotypes and possibly variants thereof of HAV are circulating the community compared to the genotypes present in water and food sources.

To identify the different strains of HAV circulating in SA, detection and characterisation of HAV strains was performed on clinical and environmental sources. A total of 117 clinical specimens and 80 water samples (irrigation, surface and wastewater) and 20 fresh produce (tomatoes, lettuce, cabbage) samples were analysed. Samples were collected in SA and neighbouring countries. Hepatitis A virus was detected in 78% of the clinical specimens and in only 46% of the water samples. None of the fresh produce analysed in the study tested positive for HAV even though some of the produce were irrigated with HAV contaminated water. Molecular characterisation of the detected strains revealed the presence of genotype IB in SA. The SA strains have a unique nucleic acid sequence, namely a R298K mutation over the VP1 region and R71S substitution over the VP1/P2B junction, which distinguishes them from strains detected in the rest of the world and even in neighbouring countries. The characterised strains were also analysed for the presence of previously isolated antigenic escape mutants and/or novel ones. One possible vaccine escape mutant, with a I179T

substitution next to the neutralisation residue Y181 in the VP1 region, was identified. A variety of strains with mutation(s) at or around the neutralisation sites were also identified as well as circulation of quasi species among clinical HAV strains. The study revealed the circulation of a potential new HAV genotype among the SA simian population. The data provided by the study stresses the need to implement a proper surveillance system of the circulation of HAV strains in SA.

9. **Aileen Naidoo (2012) (MSc Microbiology) "Impact of irrigation water on the quality of fresh produce"**

Abstract: The consumption of minimally processed fresh fruit and vegetables has increased over the past years, mostly because of consumers awareness that fresh produce serves as a good source of vitamins, minerals and fibre. Although fresh produce is important for the human diet it may provide an optimal environment for the growth and proliferation of pathogenic microorganisms, from cultivation to processing. Several outbreaks of disease, associated with the consumption of fresh produce, have been reported worldwide. In addition, fresh produce can become contaminated by heavy metals imposing a public health concern. One of the major sources of contamination is irrigation water, as it may contain pathogens and heavy metals from upstream operations. Irrigation water has been previously shown to be associated with the contamination of fresh produce. Therefore the objective of this study was to evaluate the microbial- and heavy metal-content of irrigation water used by local farmers in KwaZulu-Natal (KZN) over a 12-month period, in order to establish a link between the water quality and the safety of fresh produce, and to develop a suitable method to reduce the microbial contamination of fresh produce during both pre- and post-harvest phases. The microbial quality of the water and fresh produce samples was determined using the membrane filtration and standard spread-plate techniques, respectively. The heavy metal content of the water and fresh produce samples were analysed using inductively coupled plasma optical emission spectrophotometry (ICP-OES). Presumptive *Escherichia coli*, *Salmonella* spp., *Shigella* spp. and coliform counts in the water samples were high during the sampling period. Presumptive *E. coli* exceeded the DWAF limit of 2×103 cfu/100 ml for E. coli in irrigation water, in some instances. High counts of presumptive coliforms, *Shigella* spp. and *Campylobacter* spp. were recorded in the fresh produce, throughout the sampling period. The roots of the plant demonstrated the highest microbial and heavy metal contamination. Leafy vegetables such as spinach and lettuce were more contaminated than the other fresh produce sampled; for example, *Campylobacter* spp. exceeded 4.5×105 cfu/g in crisphead lettuce. With regard to the heavy metal content of the irrigation water and the fresh produce, mercury (Hg) exceeded the FAO and WHO limit of 0.001 mg/L, throughout the sampling period, with the highest concentration of 0.057 mg/L obtained from irrigation water. Since the concentrations of Hg in both the irrigation water and fresh produce were the highest during the same period, such as in winter, a clear link can be seen between the irrigation water and fresh produce. The method used during the pre-harvest phase, in order to reduce pathogens from produce, was the effect of Pseudomonas aeruginosa on the uptake of pathogens to the fresh produce. Inhibition assays were employed to determine whether *P. aeruginosa* could inhibit the pathogens (*E. coli, Listeria monocytogenes, Salmonella* spp. and *Shigella* spp.) tested. Only

L. monocytogenes was found to be inhibited by *P. aeruginosa*. A greenhouse experiment was employed to prove that *P. aeruginosa* could prevent the uptake of this pathogen, via the roots, into the fresh produce by monitoring the concentration of L. monocytogenes in the soil and fresh produce by standard spread-plating. Denaturing gradient gel electrophoresis (DGGE) was also used to monitor the populations of L. monocytogenes and *P. aeruginosa* in the soil. Colony counts of *L. monocytogenes* decreased from 6 to 3.5 log cfu/g in the soil during the first 3 weeks of sampling. This decrease was confirmed by DGGE and suggested that this pathogen was inhibited by *P. aeruginosa* in the soil; hence, this pathogen was also not detected in the plant. During the post-harvest phase the effect of different treatment methods on the quality of the final produce was evaluated using tap water, NaCl, chlorine, hydrogen peroxide, blanching and ultra-violet (UV) light. UV light showed the most promise as the quality of this treated produce was better as compared to the other treated produce. A link between irrigation water qualities with that of produce was evident in this study as the highest microbial counts were recorded in summer for both the water and fresh produce samples. The pre-harvest method for the reduction of pathogens from the produce, which was the effect of *P. aeruginosa* on the uptake of pathogens to the produce, was limited as this organism had only inhibited *L. monocytogenes*, of the pathogens tested. Of the post-harvest treatment methods, UV treatment had caused the highest reduction in the microbial load of the fresh produce, with tap water treatment aiding in the survival of these presumptive pathogens. The presence of *P. aeruginosa* and the use of UV light in reducing microbial counts on fresh produce had both shown promise in this study. However, further studies need to be employed in order to optimise these methods before application. In addition, irrigation water should be routinely monitored and properly decontaminated, if necessary, to prevent the transmission of food-borne pathogens to crops. This may curb the problem of food-borne associated disease outbreaks world-wide as irrigation water has been shown, by the current study, as a link to the contaminated state of fresh produce.

Doctorate study completed

Stacey Collignon (2013) (PhD Microbiology) "Risk assessment of *Escherichia coli* O157:H7, *Listeria monocytogenes Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Staphylococcus aureus* within a stone fruit production environment"

Abstract: Food safety has become an increasing concern for producers, retailers and consumers and the risks associated have become a reality for all stakeholders in the supply chain. International and voluntary standards implemented by retailers and importing countries aim to assure the safety of all fresh produce for their consumers. Therefore food safety assurance is now more than ever before an important aspect in food production and processing. Fresh produce can become adulterated at any point from the farm to the table. Producers however remain responsible for produce throughout the supply chain until the product reaches the consumers. Recently the growing number of recorded foodborne pathogen scares from fresh produce in the United States of America and the European Union has shifted the focus to the country of origin and its food safety standards, regulation and control. Fruit exported from developing countries are thought to pose a greater threat due to the use of contaminated water and perceived unhygienic production. These perceived issues

could manifest as a barrier to trade and therefore it is essential that the South African stone fruit industry can show compliance with international standards and provide evidence that fruit produced in the country is safe. Food safety assurance is an integral part of market access and sustained growth of the South African fruit industry. The aim of this study was to determine the hazards and critical control points which represent areas of risks and to determine the level of risk. This requires development of an accurate and sensitive detection method for foodborne pathogens of concern. Knowing the critical risks allow for effective management procedures to be developed to minimise the risk. A second aim of this study was to determine how the selected four important foodborne pathogens grow and/or survive under various temperature, nutrient and timeline conditions. Compiling all the data provided for a semi-quantitative risk assessment that allowed for the rating of the potential risk as high, medium or low, based on scientific evidence. The horticultural safety management systems (HSMS) of the four primary producers evaluated in this study were used as self-assessment tool to make sound recommendations to improve safety procedures and practices and thereby providing a food safety assurance framework.

Salmonella Typhimurium was not found to be a hazard or risk on peaches or plums as the pathogen was not detected from any of the over 1000 samples processed in this study. On the other hand, *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Staphylococcus aureus* were all three detected from either fruit, environmental or water samples from three of the four farms. However, these pathogens could only be considered low risk because *E. coli* O157:H7, *L. monocytogenes* and *S. aureus* were not found to survive on fruit surfaces at high enough titres to cause disease throughout the simulated export chain. The only foodborne pathogen that was detected from all four farms was *S. aureus*, an indicator of poor personal hygiene. This organism was detected from numerous surfaces including hand and contact surfaces within the packhouses, therefore demonstrating that an improvement in facility sanitation and personal hygiene was required as was confirmed using the self-assessment tool of the HSMS. Following a semi-quantitative risk assessment it was found that the risk ranking of peaches and plums was low when considering the four foodborne pathogens in this study. In conclusion, peaches and plums exported from South Africa are considered low risk products based on scientific evidence found in this study.

Annexure III Activities and Highlights

1. **Taylor MB.** Advancing the water science curriculum and building capacity toward pathogen testing in Africa [Invited presentation]. 2015 UN-Water Annual International Zaragoza Conference: Water and Sustainable Development: From Vision to Action. 15-17 January 2015 Zaragoza, Spain. ***Travel*** & ***attendance sponsored by UNESCO.***

Taylor MB, Mans J, Murray TY, Wolfaardt M. Prevalence and molecular epidemiology of noroviruses in South Africa [Invited presentation]. 63rd Annual Meeting of the American Society of Tropical Medicine and Hygiene 2-6 November 2014 Marriot Hotel, New Orleans, Louisiana, USA. ***Travel & attendance sponsored by Bill & Melinda Gates Foundation.***