Characterization of bacterial pathogens in rural and urban irrigation water

[Short title: Bacterial pathogens in irrigation water]

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ABSTRACT

The study aimed to compare the bacteriological quality of an urban and rural irrigation water source. Bacterial counts, characterization, identification and diversity of aerobic bacteria were determined. *Escherichia coli* isolated from both sites was subjected to antibiotic susceptibility testing, virulence gene (*Stx1*/*Stx2* and *eae*) determination and $(GTG)_5$ Rep-PCR fingerprinting. Low mean monthly counts for aerobic spore formers, anaerobic spore formers and *Staphylococcus aureus* were noted although occasional spikes were observed. The most prevalent bacterial species at both sites were *Bacillus* spp., *E. coli* and *Enterobacter* spp. Additionally, *E. coli* and *Bacillus* spp. were most prevalent in winter and summer respectively. Resistance to at least one antibiotic was 84% (rural) and 83% (urban). Highest resistance at both sites was to cephalothin and ampicillin. Prevalence of *E. coli* possessing at least one virulence gene (*Stx1*/*Stx2* and *eae*) was 15% (rural) and 42% (urban). All (rural) and 80% (urban) of *E. coli* possessing virulence genes showed antibiotic resistance. Complete genetic relatedness (100%) was shown by 47% of rural and 67% of urban *E. coli* isolates.

Results from this study show that surface irrigation water sources regardless of geographical location and surrounding land-use practices can be reservoirs of similar bacterial pathogens.

Key words | antibiotic resistance, bacterial pathogens, *E. coli*, irrigation water, $(GTG)_5$-Rep-PCR, virulence

ABBREVIATIONS

CDC	Centers for Disease Control and Prevention
CSIR	Council for Scientific and Industrial Research
DWAF	Department of Water Affairs and Forestry
SABS	South African Bureau of Standards
ISO	International Standards Organization
SANS	South African National Standards
EPA	Environmental Protection Agency
ACC	aerobic colony counts
AnSF	anaerobic spore formers
ASF	aerobic spore formers
FC	faecal coliforms
IE	intestinal enterococci

INTRODUCTION

Agriculture uses the bulk of fresh water requirements in the world with more than 70% used for irrigation (Gerba & Choi 2009; Ijabadeniyi & Buys 2012). Similarly, in South Africa, most water requirements are provided by surface water sources with most of it used for irrigation (Department of Water Affairs (DWAF) 2008). The heavy dependence on surface water sources is further exacerbated by lack of reliable rainfall patterns and arid/semi-arid

climatic conditions. Studies have reported on the deteriorating bacteriological quality of surface water bodies around the world (Lu *et al*. 2004; Jiang *et al*. 2007; Al' saed 2007; Gerba & Choi 2009; Pachepsky *et al*. 2011; Walters *et al*. 2011). Similarly, in South Africa, this deteriorating trend has been noted in surface irrigation water sources (Olaniran *et al*. 2009; Ijabadeniyi *et al*. 2011; Gemmell & Schmidt 2012). Faecal contamination from human and animal sources has been linked to high prevalence of these pathogens (Jan & Sadowsky 2007). High prevalence of bacterial pathogens within irrigation water sources increases the risk of contaminating irrigated produce which may compromise food safety and public health.

High resistance to antibiotics in *E. coli* has previously been noted in South African river sources although low prevalence of virulence genes was observed (Obi *et al*. 2004; Olaniran *et al*. 2009). Complementing phenotypic bacterial characterization, such as antibiotic resistance as well as toxin production with genotypic analysis provides more reliable information relating to contamination and source tracking (bacterial source tracking). This is because the genotype is less variable in comparison to phenotypic characters. Vantarakis *et al*. (2005) combined phenotypic (antibiotic resistance) and genotypic (random amplified polymorphic DNA (RAPD-PCR) methods in a study aimed at differentiating *E. coli* from human and animal sources.

The geographical location of a surface water source may also influence its risk to pathogenic bacterial contamination. For instance, in South Africa, increasing rates of urbanization, industrialization and population growth have led to pollution (Ijabadeniyi & Buys 2012). Additionally, water sources flowing through urban compared to rural areas may be exposed to more sources of pathogenic bacterial contamination resulting from informal settlements and failing sewage treatment works (Ijabadeniyi & Buys 2012). Walters *et al*. (2011) noted high prevalence of *Salmonella* spp. and faecal indicator bacteria at points close to an urban area. Additionally, Burnes (2003) and Da Silva *et al*. (2011) noted high levels of human *E. coli* in urban water sheds. However, studies have also reported on high bacterial contamination in water sources located in rural areas (Obi *et al*. 2002, 2004; Du Preez *et al*. 2008; Ijabadeniyi *et al*. 2011). Therefore, this study aimed at comparing prevalence of bacterial foodborne pathogens in two irrigation water sources within South Africa located in predominantly rural (the Loskop canal) and urban (the Skeerpoort river) areas, respectively. In addition, the study aimed at characterizing *E. coli* isolated from each irrigation water source based on antibiotic resistance, toxin production and genotypic relatedness.

METHODS

Site description

Irrigation water was collected from the Skeerpoort river (North West Province) and the Loskop canal (Limpopo Province) (Figure 1). The Skeerpoort river is downstream of the Hartbeespoort dam, a major tourist location surrounded by an urban settlement. The Loskop canal carries irrigation water throughout the Loskop irrigation scheme which is located in a predominantly rural area.

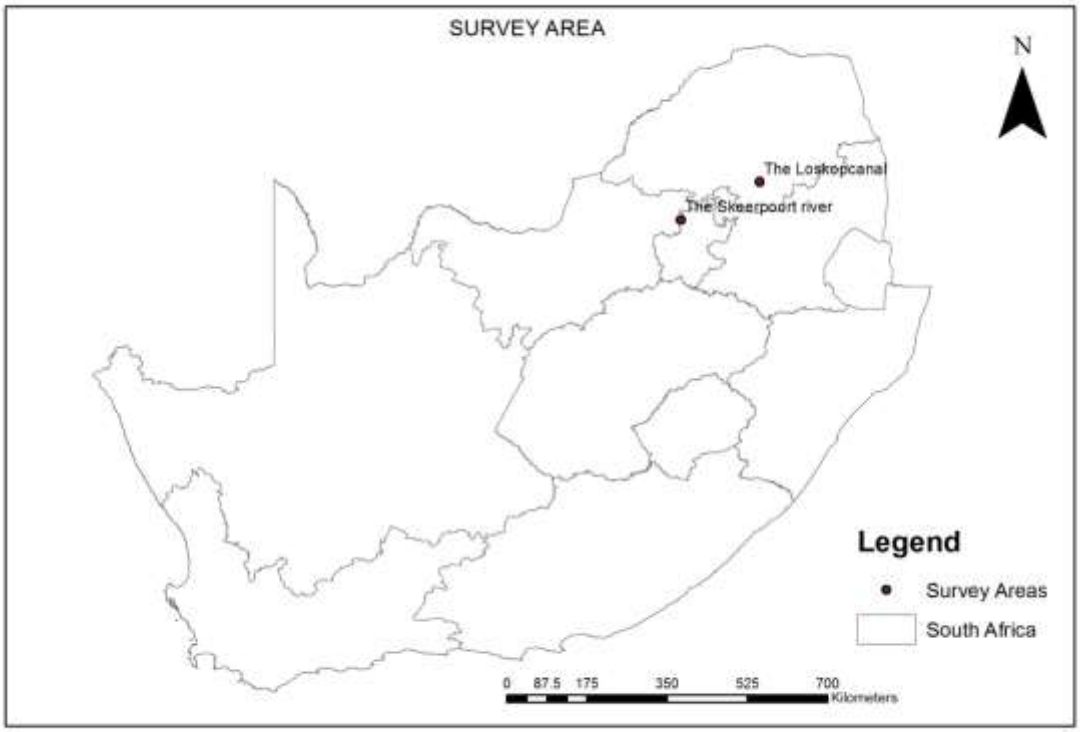

Figure 1 | Map showing sampling locations for irrigation water collected from Loskop canal and the Skeerpoort river.

Water sampling

One water sample (500mL) was collected monthly from the Loskop canal and the Skeerpoort river for 10 months from January to October, 2011. Water samples were collected aseptically in sterile plastic bottles for physico-chemical and bacteriological analyses and transported to the Department of Food Science, University of Pretoria and analysed within 6 hours.

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

Microbiological analysis involved screening for bacterial contaminants (Table 1) in irrigation water from the Loskop canal and the Skeerpoort river.

Analysis of water samples

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*, *S. aureus*, *Listeria monocytogenes* and *Salmonella* spp.) were enumerated and identified (Table 1). Direct plating (0.1 mL) of serially diluted water samples onto respective enumeration and identification agar was done for ACC, AnSF, ASF and *S. aureus*. For TC and FC, 1 mL of sample was inoculated into tubes containing 10 mL of Lauryl Tryptose Broth (Oxoid Ltd, Basingstoke, Hampshire, UK), Brilliant Green Bile agar (Oxoid Ltd) and EC Broth (Oxoid Ltd) using the most probable number (MPN) method (Table 1). To identify presumptive *E. coli*, positive tubes containing EC broth were streaked onto Eosin Methylene Blue Agar (Levine) (Oxoid Ltd). Enrichment of *L. monocytogenes* was done using Half Fraser and Full Fraser media (Oxoid Ltd) with contents from each medium subsequently streaked onto Oxford and Palcam selective agars (Oxoid Ltd) after incubation. One millilitre (1 mL) of sample was added to 9 mL of Half Fraser broth incubated at 30 °C for 24 h and 0.1 mL of this incubated mixture was added to 10 mL of Full Fraser incubated at 35 °C for 48 h. Lastly, enrichment of *Salmonella* spp. was done using buffered peptone water (Biolab Diagnostics (Pty) Ltd, Wadeville Gauteng, South Africa), Salmonella enrichment broth (Oxoid Ltd) and Selenite Cystein broth (Oxoid Ltd). Twenty-five millilitres (25 mL) of water sample was added to 225 mL of buffered peptone water and incubated for 24 h. The enriched sample was placed into Salmonella enrichment broth (1 mL of sample into 10 mL of medium) and Selenite Cysteine broth (10 mL of sample in 100 mL of medium) and incubated at 42 °C for 24 h and 35 °C for 48 h, respectively. The contents of both selective media were streaked onto Phenol Red Brilliant Green agar and XLD agar (Oxoid Ltd).

Table 1 | Methodology for detection of bacterial organisms in irrigation water

Organism	**Media**	**Temperature/Time of incubation**	**Company**	***Reference**
Aerobic colony count (ACC)	Standard Plate Count Agar	30 °C for 48 to 72 h	(Oxoid Ltd, Basingstoke Hampshire, UK)	SABS ISO 4833 (1991)
Aerobic spore formers (ASF)	Trypticase Soy Agar	35 °C for 48 h	(Biolab Diagnostics (Pty) Ltd, Wadeville Gauteng, South Africa)	(MFLP-44) (Health Canada, 1998)
Anaerobic spore formers (AnSF)	Trypticase Soy Agar	35 °C for 48 h	(Biolab)	
Total coliforms	Lauryl Tryptose (LST) Broth	35 °C for 24 to 48 h	(Oxoid)	MFHB19 (Health Canada, 2002)
Faecal coliforms	Brilliant Green Bile 2% Broth (BGLB)	35 °C for 24 to 48 h	(Oxoid)	
	E.C Broth	35 °C for 24 to 48 h	(Oxoid)	
E. coli	Eosin Methylene Blue Agar (Levine) (EMB)	35 °C for 24 h	(Oxoid)	
	E. coli/chromogenic Medium	35 °C for 18 to 24 h	(Oxoid)	
	Agar Chrom ID ™ 0157 H7 (O157 ID-F)	35 °C for 18 to 24 h	(BioMerieux, Marcyl-l' Etoile, France)	
Intestinal enterococci	Slanetz and Bartley Medium	35 °C for 44 h	(Oxoid)	SANS ISO 7899-2 (2004)
	Bile Esculin Agar	44 °C for 2 h	(Oxoid)	
S. aureus	Baird-Parker Agar	35 °C for 48 h	(Oxoid)	SABS ISO 6888-1 (1999)
[§]*Salmonella* spp.	Brilliance™ Salmonella Agar Base	35 °C for 24 to 48 h	(Oxoid)	SABS ISO 6579 (2003)

	Rapid Salmonella	35 °C for 24 to 48 h	(Biorad, Marnes-la-Coquette France)	
†*L. monocytogenes*	Listeria Selective Agar (Oxford formulation)	35 °C for 48 h	(Oxoid)	SABS ISO 11290-1 (1996)
	Listeria Selective Agar (Palcam Selective Supplement)	35 °C for 48 h	(Oxoid)	

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

* SABS, South African Bureau of Standards; ISO, International Standards Organization; SANS, South African National Standard; MFHB19, Health Products and Food Branch, Methods for the microbiological analysis of foods: determination of coliforms, faecal coliforms and *Escherichia coli* in foods; MFLP44, Laboratory methods for the microbiological analysis of foods; determination of aerobic and anaerobic endospore formers.

Characterization and identification of most prevalent aerobic bacteria

To determine the most prevalent aerobic bacterial species within both irrigation water sources, for each sample analysed, three different colonies (visual discrimination) were randomly picked from the highest dilution standard plate count agar petri-dish. Ten samples were analysed monthly for 10 months. Gram stain and the catalase test were done. Sixty (60) bacterial isolates were identified from the Loskop canal (30) and the Skeerpoort river (30). Isolates were identified to species level with the Omnilog® Data Collection Software Identification System version 2.1 (Biolog Inc. Hayward, California) following prior isolation on plate count agar (Oxoid) and subsequently on Biolog Universal Growth medium (BUG) (Biolog).

Antibiotic resistance in *E. coli*

A total of 31 *E. coli* isolates from the Loskop canal (19) and the Skeerpoort river (12) were analysed. From the total group of isolates, 13 were isolated on Eosin Methylene Blue agar (Levine) (EMB) (Oxoid) and identified with selective *E. coli*/chromogenic medium (Oxoid) and Agar Chrom ID™ 0157 H7 (O157 ID-F) (BioMerieux, Marcyl-l' Etoile, France) after to isolation from the most probable number method (Table 1). Additionally, 18 isolates (selected from aerobic plate counts) were identified using the Omnilog® system referred to following isolation on plate count agar (Oxoid) and subsequently Biolog Universal Growth medium (BUG) (Biolog). Eleven (11) antibiotics (Oxoid) at single concentrations were selected according to Da Silva *et al*. (2011). Antimicrobial susceptibility was done using multiple antibiotic resistance (MAR) analysis (Vantakaris *et al*. 2006). 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 genes in *E. coli*

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).

$(GTG)_5$-Rep-PCR fingerprinting of *E. coli*

The same isolates used for antibiotic resistance and virulence gene analysis were subjected to $(GTG)_5$-Rep-PCR**.** 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 1,500 bp (Invitrogen) was used to estimate the length of amplicons.

Statistical analysis

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

The resulting fingerprints from $(GTG)_5$-Rep-PCR were analysed using the GelCompare III version 5.10 (Applied Maths, Saint-Marten-Latem, Belgium) software package. The similarity among digitized bands was calculated using the Pearson correlation, and an average linkage (UPGMA or unweighted pair group method with arithmetic averages) dendrogram was derived from the profiles.

RESULTS

Physicochemical quality indicators

The pH ranged from 5.4 to 9.9 and 7.9 to 9.2 in the Loskop canal and the Skeerpoort river, respectively. The pH in the Loskop canal exceeded South African national guidelines (DWAF, 1996) for irrigation water (6.5 to 8.4) twice during the 10 month study. Water temperature in the Loskop canal ranged from 8.5 to 20.5 °C with the lowest and highest noted in September and February, respectively. Temperatures in the Skeerpoort river ranged from 10.7 to 26.0 °C

with the lowest and highest noted in May and January, respectively. Higher mean monthly rainfall was noted in areas around the Skeerpoort river (74.7 mm) than the Loskop canal (0.1 mm) (personal communication: Lucky Dlamini, South African Weather Service, July 2012).

Bacterial indicators

Mean monthly aerobic colony counts (ACC) in the Loskop canal and the Skeerpoort river were 3.2 and 3.4 $\log_{10}$ cfu/mL, respectively (Table 2). Mean monthly anaerobic spore formers (AnSF) and aerobic spore formers (ASF) ranged from 1.8 to 2.5 $\log_{10}$ cfu/mL in both water sources. However, high counts (5.1 $\log_{10}$ cfu/mL) of ASFs were noted in the Loskop canal in April and October (Figure 2).

Additionally, high counts of AnSF (5.3 $\log_{10}$ cfu/mL) were noted in June and October in the Skeerpoort river (Figure 3). Low mean faecal coliforms (FC) (1.3 $\log_{10}$ cfu/mL) were noted in the Loskop canal and the Skeerpoort river (Table 2). The FC surpassed South African national guidelines (10 to 1000 FC per 100mL) for irrigation water once (March) in the Skeerpoort river (Figure 3).

Counts of intestinal enterococci (IE) ranged from 0.1 to 0.4 $\log_{10}$ cfu/mL in the Loskop canal and the Skeerpoort river (Table 2). Prevalence of *E. coli* was 40% at both irrigation water sites (Figures 2 and 3). Other than May, *E. coli* was isolated in January, April and August in the Loskop canal and March, September and October in the Skeerpoort river. *Escherichia coli* was isolated from the Loskop canal and the Skeerpoort river in months when the highest FC counts were recorded (Figures 2 and 3). *Escherichia coli* O157:H7 was isolated in September in both water sources as well as in October in the Skeerpoort river. *Staphylococcus aureus* was noted in a few months at both irrigation water sites. In the Loskop canal, *S. aureus* was noted in March, April, August and September (Figure 2). In the Skeerpoort river, *S. aureus* was noted in February, March, April and May (Figure 3). Additionally, the pathogen was noted in three (the Skeerpoort river) and four (Loskop canal) months during the ten-month study (Figures 2 and 3). However, when *S. aureus* was noted, counts ranged from 1.6 to 3.0 $\log_{10}$ cfu/mL in the Loskop canal and 0.6 to 2.0 $\log_{10}$ cfu/mL in the Skeerpoort river (Figures 2 and 3). *Salmonella enterica* subsp. *salamae* (typed as *Salmonella* II 13, 22, 23) was isolated from the Loskop canal (Table 2).

Diversity of aerobic bacterial species

Seven bacterial genera/species were isolated from the Loskop canal. They included *Bacillus* spp., *Enterobacter* spp., *E. coli*, *Klebsiella* spp., *Kluyvera ascorbata*, *Enterococcus gallinarum* and *Serratia marcescens* subsp. *marcescens* (Table 3). Eight genera/species were

isolated from the Skeerpoort river. They included *E. coli*, *Bacillus* spp., *Enterobacter* spp., *Klebsiella* spp., *Raoultella* spp., *Burkholderia* spp., *Buttiauxella* spp. and *Salmonella* spp. (Table 3). *Bacillus* spp., *E. coli and Enterobacter* spp. were the most prevalent bacteria in the Loskop canal and the Skeerpoort river (Figures 4 and 5). *Escherichia coli* was most prevalent in winter and *Bacillus* spp. in summer at both irrigation water sites. Low prevalence (3.4%) for *Kluyvera ascorbata*, *K. oxytoca*, *Enterococcus gallinarum*, *K. pneumoniae* subsp. *pneumoniae* and *S. marcescens* subsp. *marcescens* was noted in the Loskop canal (Figure 4). Similarly, low prevalence (3.3%) of *Salmonella* spp., *Klebsiella* spp., *Raoultella* spp., *Burkholderia* spp., *Buttiauxella* spp. was noted in the Skeerpoort river (Figure 5).

Table 2 | Physico-chemical parameters, bacterial counts and incidence of bacterial contaminants in irrigation water from the Loskop canal and the Skeerpoort river for samples (n = 20) collected monthly over 10 months

				Indicator parameters (log_{10} cfu/mL, g)						% samples positive for bacterial contaminants	
	No. of samples	**Temp (°C)**	**pH**	**Aerobic colony count**	**Anaerobic spore formers**	**Aerobic spore formers**	***S. aureus***	**IE**	**FC**	***E. coli***	***Salmonella* spp.**
Loskop canal[1]	10	15.4 ± 3.7	7.4 ± 1.1	3.2 ± 0.7	1.8 ± 1.9	2.1 ± 2.1	0.9 ± 1.2	0.1 ± 0.2	1.3 ± 1.0	40	10
Skeerpoort river	10	18.2 ± 6.2	8.4 ± 0.4	3.4 ± 0.6	2.5 ± 1.8	2.5 ± 1.2	0.5 ± 0.8	0.1 ± 0.4	1.3 ± 1.1	40	ND
Average	20	16.8 ± 2.0	7.9 ± 0.7	3.3 ± 0.1	2.2 ± 0.5	2.3 ± 0.3	0.7 ± 0.3	0.1 ± 0.0	1.3 ± 0.0	40	5

S. aureus, *Staphylococcus aureus*;

IE, intestinal enterococci;

FC, faecal coliforms (log_{10} MPN/100mL, g);

[1] *Salmonella enterica* subsp. *salamae* was isolated from the Loskop canal;

±, standard deviation;

ND, not determined

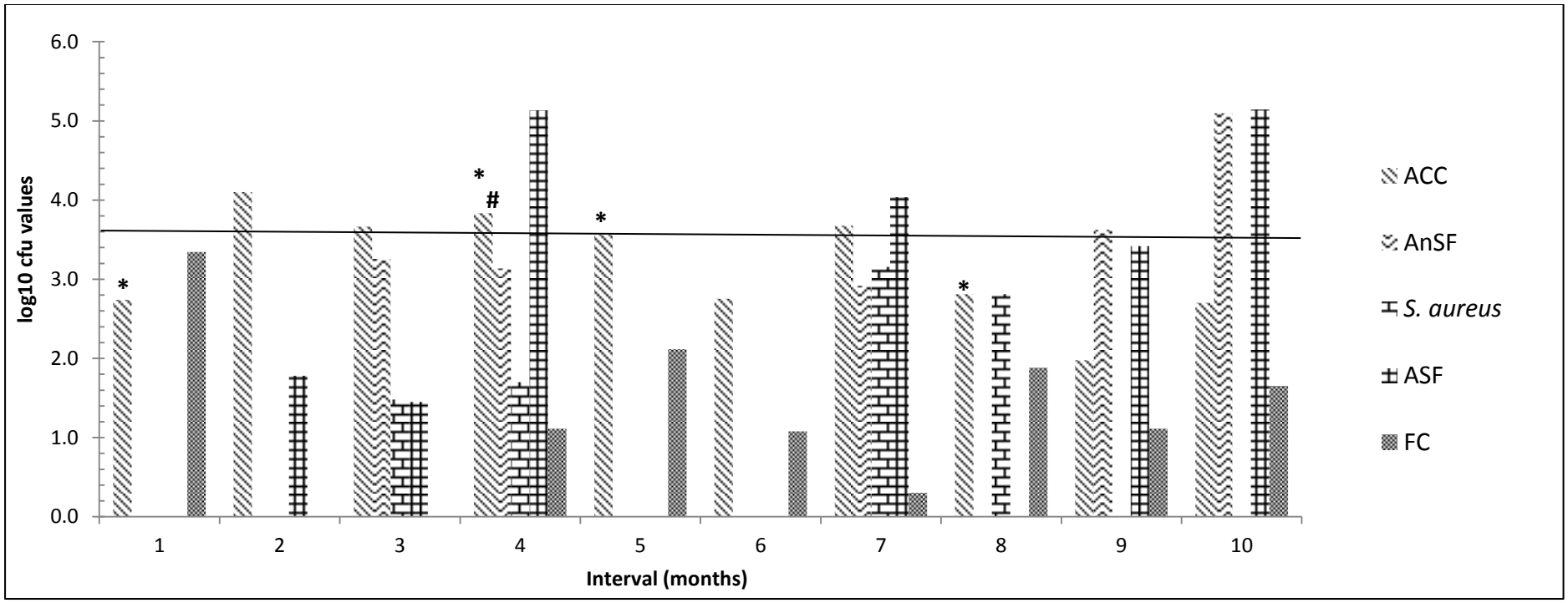

Figure 2 | Bacterial counts and incidence of *E. coli* and *Salmonella* spp. in irrigation water from the Loskop canal for samples (n = 10) collected monthly over 10 months. *, Interval positive for *E. coli*, #, interval positive for *Salmonella enterica* subsp. s*alamae*; ACC, Aerobic colony count; AnSF, anaerobic spore formers; ASF, aerobic spore formers, *S. aureus*, *Staphylococcus aureus*; FC, faecal coliforms (log_{10} MPN/100 mL). The horizontal line represents upper limit for faecal coliforms in South African irrigation water (Department of Water Affairs and Forestry (DWAF) 1996)

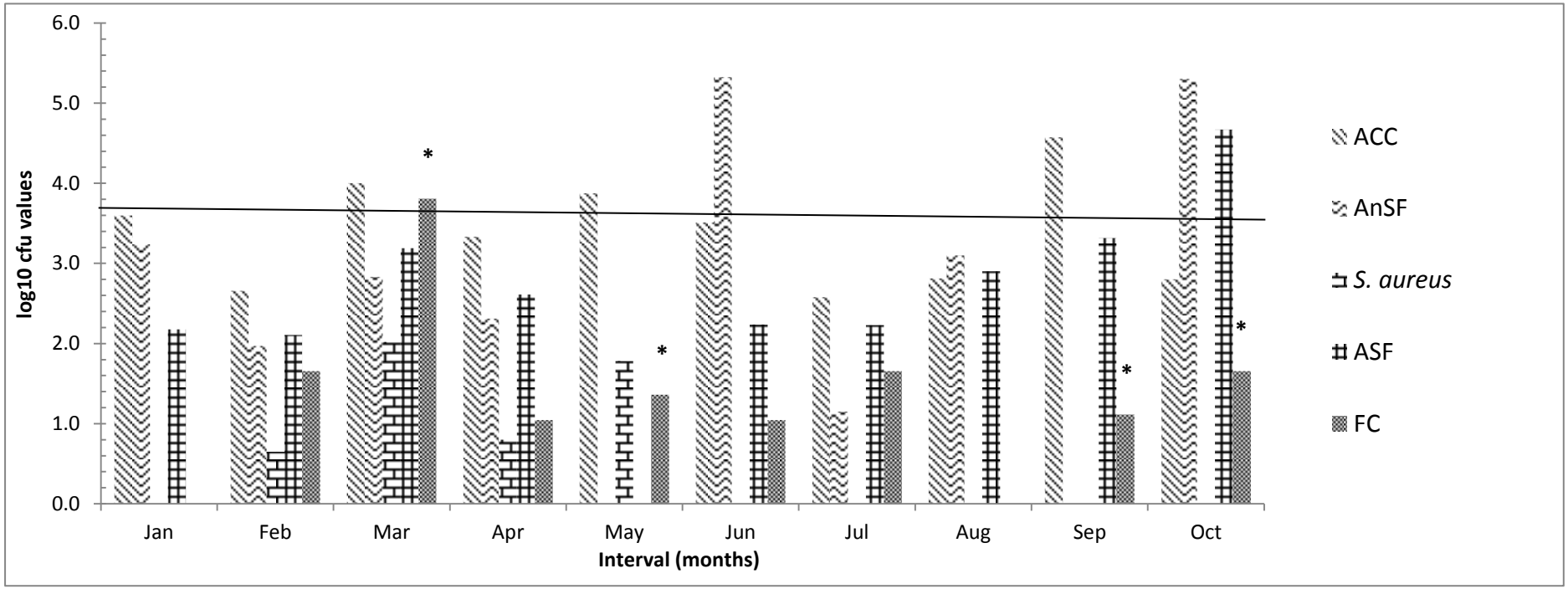

Figure 3 | Change of bacterial counts and incidence of *E. coli* with time in irrigation water from the Skeerpoort river for samples (n = 10) collected monthly over 10 months. *, interval positive for *E. coli*; ACC, Aerobic colony count; AnSF, anaerobic spore formers; ASF, aerobic spore formers; *S. aureus*, *Staphylococcus aureus*; FC, faecal coliforms (log_{10} MPN/100 mL)). The horizontal line represents upper limit for faecal coliforms in irrigation water (DWAF 1996).

Table 3 | Prevalence of aerobic bacteria in irrigation water from the Loskop canal and the Skeerpoort river for isolates (n = 60) collected monthly over 10 months

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

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

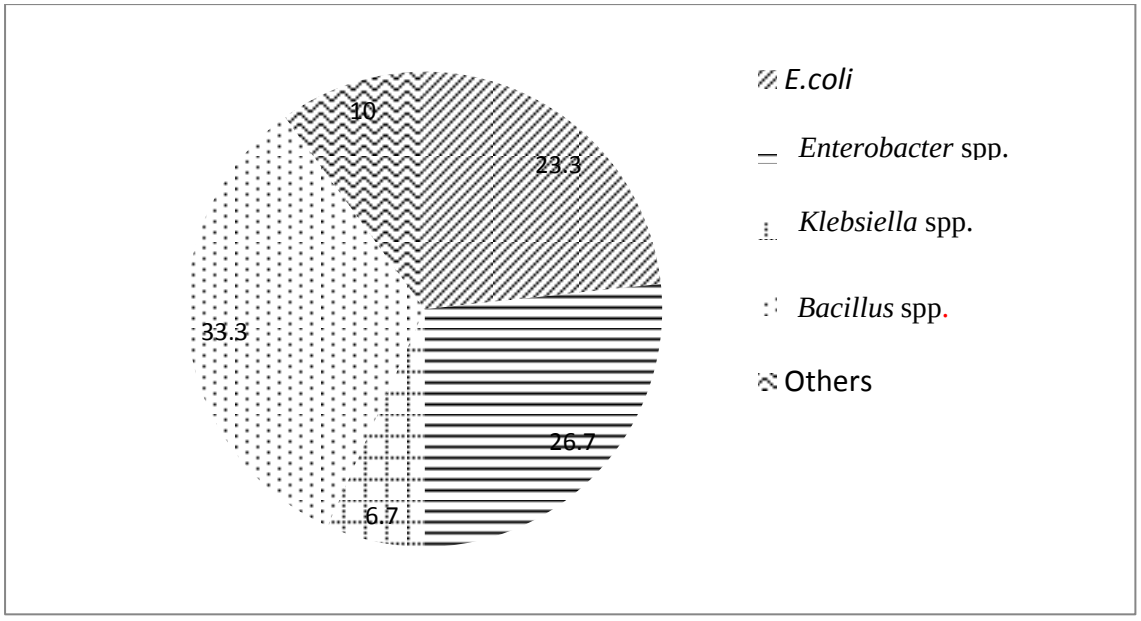

Figure 4 | Prevalence of aerobic bacteria (n = 30) in irrigation water from the Loskop canal for samples (n = 10) collected monthly over 10 months. Others: *Kluyvera ascorbata* and *Serratia marcescens* subsp. *marcescens*

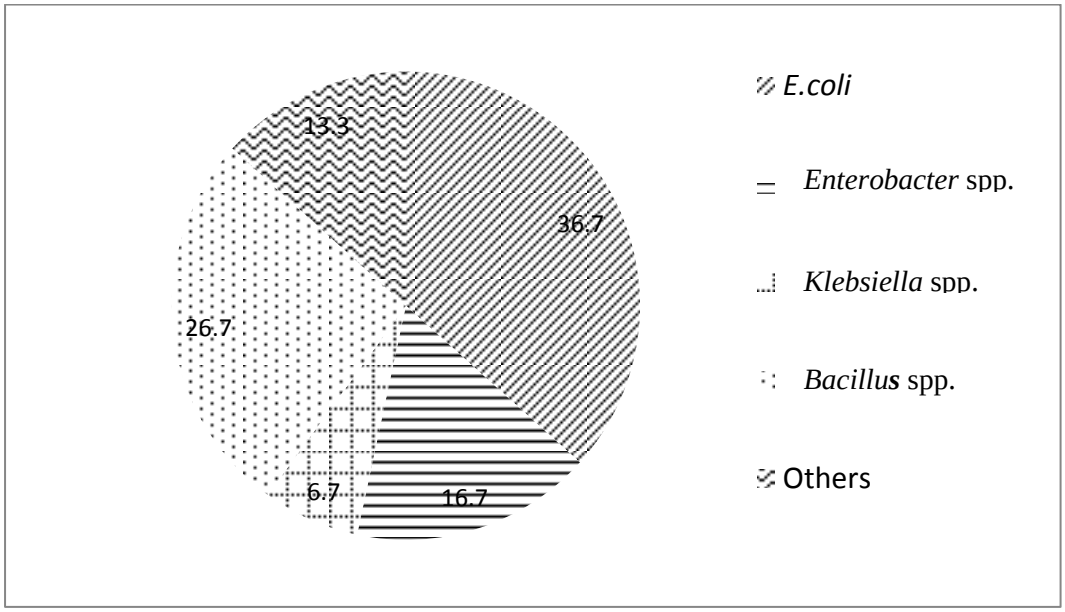

Figure 5 | Prevalence of aerobic bacteria (n = 30) in irrigation water from the Skeerpoort river for samples (n = 10) collected monthly over 10 months. Others: *Raoultella terringena*, *Burkholderia caprophylil*, *Buttiauxella agrestis* and *Salmonella enterica*

Antibiotic resistance in *E. coli*

Escherichia coli from the Loskop canal was resistant to nitrofurantoin, ampicillin, nalidixic acid, gentamicin, oxytetracycline, amikacin, cephalothin, neomycin and amoxicillin. Similarly, *E. coli* from the Skeerpoort river showed resistance to nalidixic acid, norfloxacin, neomycin, amoxycillin, ampicillin, cephalothin and oxytetracycline. Resistance of *E. coli* to at least one antibiotic was 84.2% and 83.3% in the Loskop canal and the Skeerpoort river, respectively. Highest resistance to single antibiotics in both water sites was to cephalothin and ampicillin (Table 4). Resistance to more than one antibiotic in the Loskop canal and the Skeerpoort river was 42.1% and 50%, respectively. However 5.3% of isolates in the Loskop canal and 33.3% in the Skeerpoort river were resistant to more than two antibiotics.

There was a significant difference ($p \leq 0.05$) in resistance to antibiotics in *E. coli* from the Loskop canal and the Skeerpoort river. *Escherichia coli* in the Loskop canal was susceptible to norfloxacin and chloramphenicol while *E. coli* from the Skeerpoort river was susceptible to amikacin, gentamicin, chloramphenicol and nitrofurantoin. Eight antibiotic-resistant patterns in the Loskop canal compared to six patterns in the Skeerpoort river (Table 5). *Escherichia coli* from the Skeerpoort river showed more resistance to multiple antibiotics (Table 5). *Escherichia coli* from the same irrigation water source showed close phenotypic relatedness based on resistance to antibiotics (Figure 6). However, some isolates from different sources (7 and 18 in the Loskop canal) and (f and d in the Skeerpoort river) showed close relatedness, respectively (Figure 6).

Virulence genes in *E. coli*

In the Loskop canal, 15.7% of isolates were positive for at least one virulence gene. Additionally, 10.5% of isolates were positive for *stx1*/*stx2* and *eae* and 5.2% for *stx1*/*stx2* (Table 6). In the Skeerpoort river, 41.6% of isolates were positive for at least one virulence gene with 25% of them positive for *stx1*/*stx2* and *eae* (Table 6). Additionally, 8.3% of isolates were positive for only *stx1/stx2* and *eae*, respectively. All isolates having virulence genes in the Loskop canal showed antibiotic resistance while 80% of isolates possessing virulence genes in the Skeerpoort river, showed antibiotic resistance.

Genetic fingerprinting

Escherichia coli in the Loskop canal (47.3%) and the Skeerpoort river (67%) showed complete (100%) genetic relatedness (Figure 7). Additionally, 53% of isolates from the

Loskop canal and 75% in the Skeerpoort river showed relatedness higher than 80% (Figure 7).

Table 4 | Prevalence of antibiotic-resistant *E. coli* collected over 10 months in irrigation water from the Loskop canal and the Skeerpoort river

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

ND, no resistance

Table 5 | Multiple resistances to antibiotics in *E. coli* isolated over 10 months from irrigation water in the Loskop canal and the Skeerpoort river

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

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

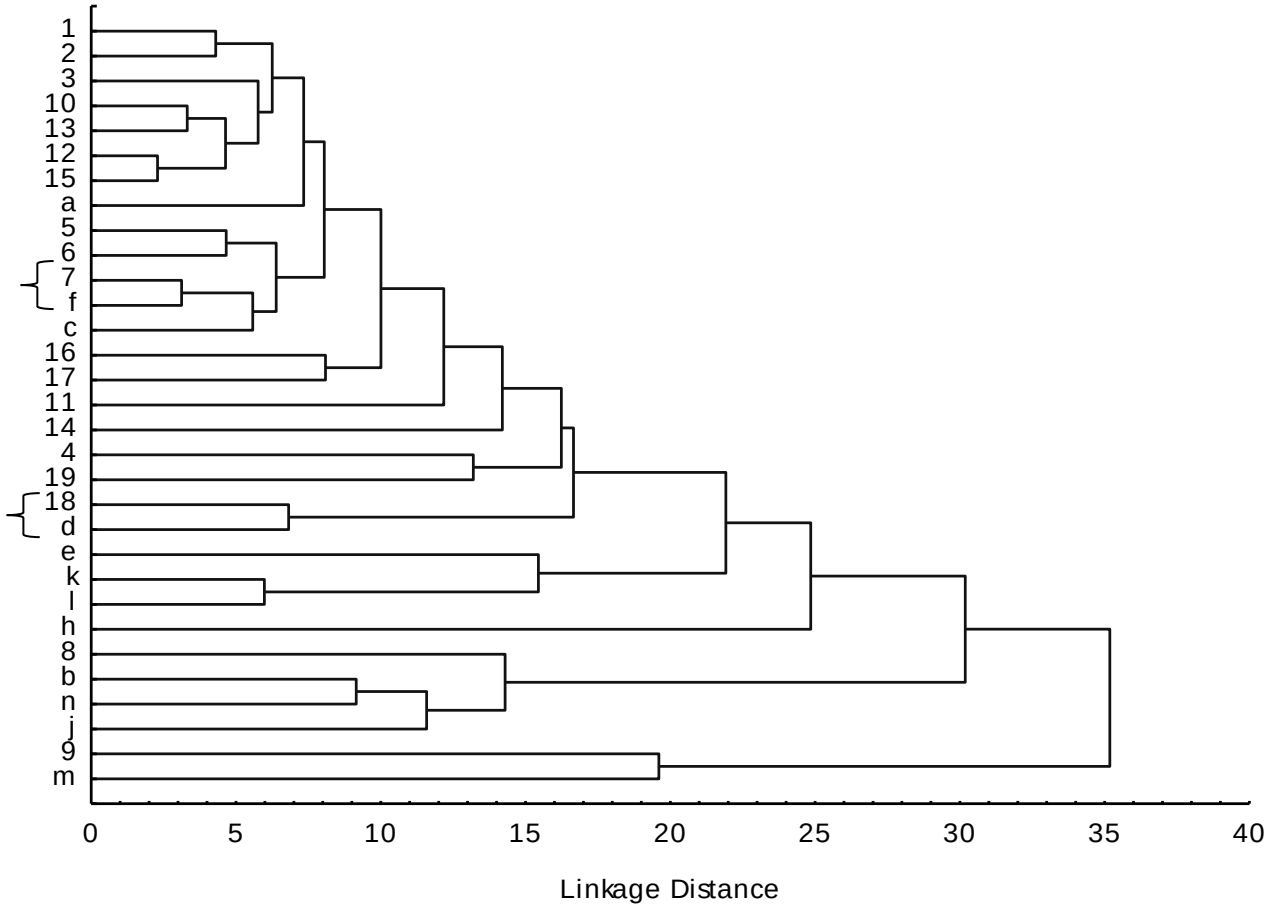

Figure 6 | Dendrogram showing cluster formation for E. coli collected over 10 months from the Loskop canal and the Skeerpoort river. Numbers represent isolates from the Loskop canal and alphabetical letters represent isolates from the Skeerpoort river. Brackets enclose closely related isolates from different sites.

Table 6 | Prevalence of virulence genes in *E. coli* isolated over 10 months from the Loskop canal and the Skeerpoort river

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

stx 1, Shigatoxin 1 gene; *stx* 2, Shigatoxin 2 gene; *eae*, Intimin gene; ND, not determined

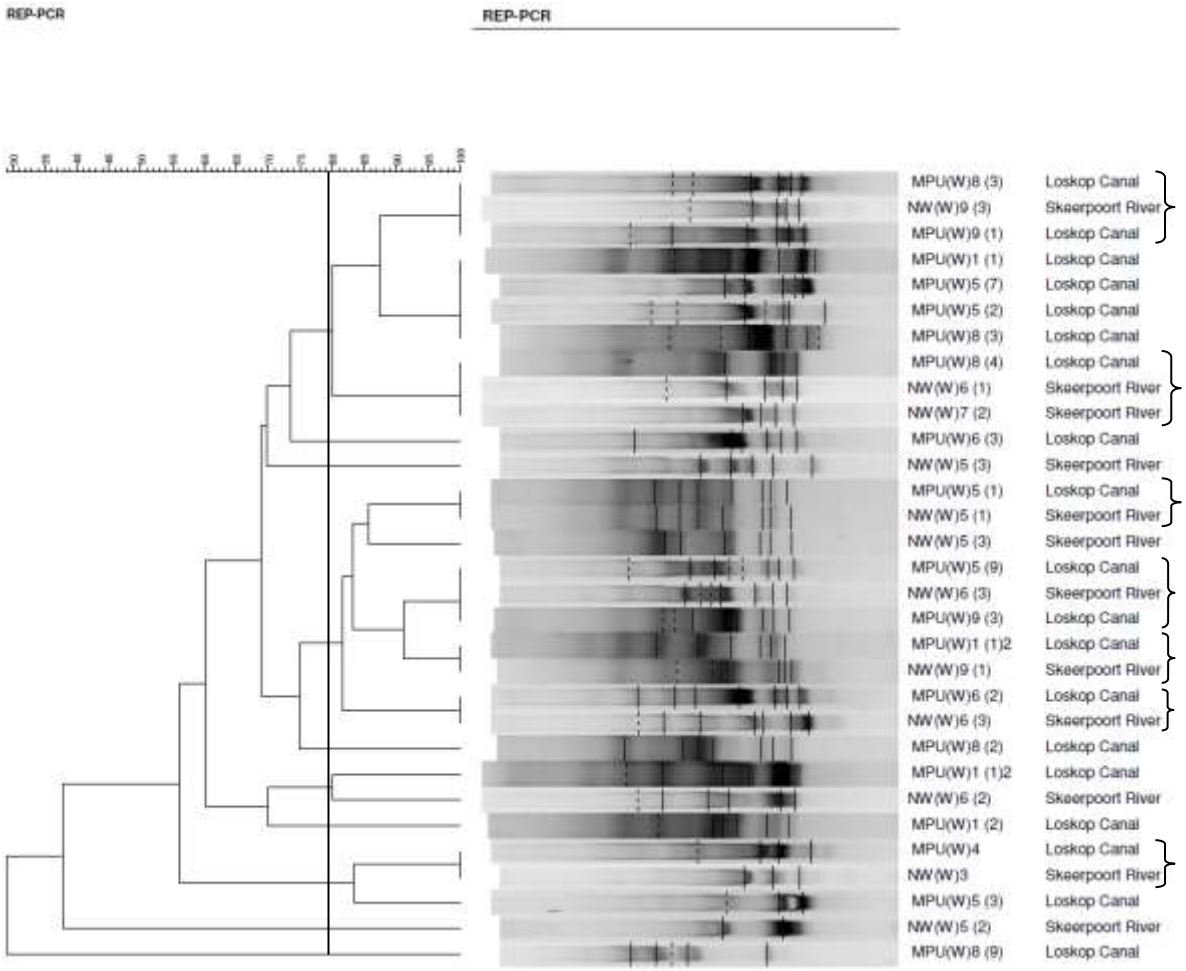

Figure 7 | Cluster analysis of $(GTG)_5$-PCR fingerprints of *E. coli* isolates collected from the Loskop canal (19) and the Skeerpoort river (12) . The vertical line depicts clusters of isolates that showed 80% similarity which was taken as the threshold for closely related isolates. Isolate codes are directly opposite the source of isolation.

DISCUSSION

Physico-chemical and bacterial indicators

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

Changes in ACC, ASF and AnSF in the Loskop canal and the Skeerpoort river which did not follow a particular trend may have been due to isolated events of pollution at different times. Bacterial indicator counts may vary as a result of availability of nutrient growth requirements, settling to sediment, chemical reactions, decay or due to seasonal changes (Pachepsky *et al*. 2011). Low mean counts for bacterial indicators in the Loskop canal may have been due to the fencing around the canal, concrete flooring and side walls which might have reduced bacterial contamination from extraneous sources such as animals and run-off. Use of a reservoir dam for collecting irrigation water in the Skeerpoort river may have protected it from contamination sources commonly associated with free flowing rivers such as runoff, animal faecal material, domestic sewage and industrial effluent. Low and sporadic counts of ACC, ASF and ASF were previously noted in the Loskop canal (Ijabadeniyi *et al*. 2011).

High FCs noted during summer when high rainfall was also noted, suggested a positive linear relationship between faecal contamination and rainfall. This was possibly due to a larger volume of run-off generated which increased contamination within the water source as well as the churning of sediment (Korajkic *et al*. 2010). High prevalence of *E. coli* noted at the two sites is indicative of possible faecal contamination and ability of the pathogen to stay viable in irrigation water during winter (Islam *et al*. 2004). This may increase the risk of possible pathogenic transfer and proliferation on irrigated produce.

Escherichia coli was isolated from irrigation water and on fresh produce irrigated with water from the Loskop canal (Ijabadeniyi *et al*. 2011) and the Skeerpoort river (Duhain 2011). Predominance of *E. coli* during winter at both sites suggested adaptation to low environmental temperatures compared to other isolated bacterial species. *Escherichia coli* has been suggested to apply a dual regulation system enabling selection and production of scarce metabolic requirements which assist and maintain growth in secondary environments such as water and the plant phyllosphere (Seurinck *et al*. 2005). Additionally, *E. coli* have petrichous pili-flagella (Welch 2006) that aid movement in liquid environments, an adaptation that may enhance survival by enabling transfer to nutrient-rich areas. These adaptations may help *E. coli* stay viable in irrigation water, increasing the likelihood of contaminating irrigated produce. High prevalence of *E. coli* has previously been noted at lower temperatures of 8 °C (Berry & Wells 2010).

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

Enterobacter spp. and *K. pneumonia* are common opportunistic pathogens. *Klebsiella pneumoniae* proliferates in the human intestines and is found in faeces (Centers for Disease Prevention and Control (CDC) 2012), therefore its presence may indicate possible faecal contamination at both irrigation water sites. This organism has been isolated from irrigation water and lettuce (Olayemi 1997) as well as from raw lettuce on sale in markets (Puspanadan *et al*. 2012). *Klebsiella pneumoniae* is usually associated with healthcare-associated infections, wounds or surgical site infections and meningitis in individuals with compromised immunity such as infants, the ill and aged (CDC 2012). This pathogen has been noted as a cause of infections among newborn babies in South Africa (Ballot *et al*. 2012) and other developing countries (Zaidi *et al.* 2009; Decré *et al*. 2011) with sources of infection linked more to the environment than maternal hygiene (Zaidi *et al*. 2009).

High prevalence of *Enterobacter* spp. (Zamxaka *et al*. 2004; Lin & Biyela 2005; Lötter 2010) and *Klebsiella* spp. (Samie *et al.* 2010) have been noted in South African water sources.

Antibiotic resistance, virulence genes and genotypic fingerprinting of *E. coli*

High resistance to cephalothin and ampicillin noted in *E. coli* isolated from the two sites, may have suggested extended exposure to these antibiotics possibly resulting from common use within the surrounding areas. Extended exposure of *E. coli* to antibiotics has been noted to increase resistance whereby resistance genes may be transferred by horizontal genetic transfer occurring on mobile genetic elements such as plasmids (Da Silva & 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 located in South Africa (Olaniran *et al*. 2009).

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

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

Antibiotic resistance can be used to determine relatedness of environmental *E. coli* as well as the source of contamination (Wiggins *et al*. 1999). Similarly, genotypic relatedness among *E. coli* isolates from the two sites suggested similar sources of contamination. This observation is in line with the results from antibiotic resistance analysis that suggested that similar sources of faecal contamination were responsible for contaminating the Loskop canal and the Skeerpoort river despite of the geographical and possible land-use difference.

Fingerprinting using $(GTG)_5$-Rep-PCR has been used to track sources of bacterial faecal contamination within water sources (Mohapatra & Mazumder 2008). The method was more resolute in discriminating *E. coli* strains from different animal sources than Rep-PCR using BOX and ERIC primers (Mohapatra & Mazumder 2008). The combination of phenotypic and genotypic methods to determine sources of bacterial contamination has been recommended because it provides a more accurate mode of assessing environmental bacterial contamination (EPA 2005).

The presence of genes associated with enterohaemorrhagic *E. coli* (EHEC) infections within *E. coli* from the two sites suggested the ability to cause shigatoxin-related human infections. Additionally, resistance to antibiotics noted within strains with virulence genes may heighten the risk and severity of illness due to the reduced effectiveness of antimicrobial therapy. Therefore, such water is unsuitable for irrigating fresh produce undergoing minimal processing (Pachepsky *et al*. 2011). Studies have reported on high resistance to antibiotics in shigatoxin producing *E. coli* (STEC) isolated from animal waste water (Schroeder *et al*. 2002; Da Silva & Medonça 2012). *Escherichia coli* coding for *stx1*, *stx2* and *eae* genes was noted as resistant to ampicillin and streptomycin (Da Silva & Medonça 2012).

CONCLUSION

This study has shown that surface irrigation water sources, regardless of geographical location and surrounding land-use practices, can be reservoirs of bacterial pathogens that may compromise the safety of irrigated minimally processed produce. Therefore, continuous and consistent monitoring of the bacteriological quality of irrigation water sources is essential in order to assess levels of pathogenic bacterial contamination, determine potential sources of contamination and provide reliable information for use in remediation.

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