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

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ABSTRACT

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

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

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

Significance and Impact of Study: Multidrug-resistant *E. coli* presence throughout spinach production emphasizes the necessity of increased surveillance of AMR in fresh produce and

the production environment within a One Health paradigm to develop AMR mitigation strategies.

Keywords: antimicrobial resistance, *E. coli*, food safety, fresh produce, irrigation water, Salmonella

INTRODUCTION

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

Surveillance of foodborne pathogens forms an important part of disease outbreak assessment and is a critical component of food safety. However, foodborne diseases in South Africa (SA) are often not reported in an epidemiological surveillance system- or are under-reported and poorly investigated (Bisholo et al., 2018; Frean, 2010). Globally, an increase in foodborne outbreaks linked to fresh produce has been reported, with leafy green vegetables in particular posing a higher risk for the consumer (World Health Organisation [WHO], 2008). Leafy green vegetables often associated with foodborne illness include spinach, lettuce and kale (Centre for Disease Control & Prevention [CDC], 2017; European Food Safety Authority [EFSA], 2018). Sources of contamination with pathogens such as *E. coli* O157:H7 or *Listeria monocytogenes* in leafy green vegetables include contaminated irrigation water, soil or processing facilities (CDC, 2020; Self et al., 2019). Specific examples in the United States of America (USA) include the 2006 multistate packaged spinach outbreak and the 2019 multistate romaine lettuce outbreak, both associated with *E. coli* O157:H7, while in 2016 a multistate outbreak in packaged leafy green salads associated with *L. monocytogenes* were reported (CDC, 2020; Jay et al., 2007; Self et al., 2019).

Irrigation water is regarded as one of the primary reservoirs, and routes of transmission, of human pathogenic bacteria onto fresh produce during primary production (Allende & Monaghan, 2015). In SA, 25%–30% of the agricultural industry relies on irrigation, with the total volume of water utilized for irrigated agriculture estimated to be between 51% and 63% of total water available in the country (Bonthuys, 2018). Sources of irrigation water include untreated or treated wastewater, surface water, borehole water from shallow- or deep groundwater and potable or rainwater (Iwu & Okoh, 2019). The water scarcity in SA has led to the use of mainly surface water for irrigation purposes in vegetable production (du Plessis et al., 2015). The microbiological quality of surface water is severely compromised due to mainly densely populated human settlements close to the surface water sources as well as mining and industry activities (du Plessis et al., 2015; Duvenage & Korsten, 2017; Iwu & Okoh, 2019; Oberholster & Botha, 2014). As fresh produce production and processing rely on potable water, increased food safety risks arise when irrigation water are increasingly being polluted (Uyttendaele et al., 2015). The frequency of fresh produce contamination, prevalence of

generic *E. coli* levels, and the presence of pathogenic foodborne bacteria in irrigation water may vary (Alegbeleye et al., 2018; Allende & Monaghan, 2015). This follows as seasonality, land use interactions (e.g. waste water treatment plants upstream of irrigation source water) and farming production practices differ (Alegbeleye et al., 2018; Allende & Monaghan, 2015).

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

However, no studies have investigated the microbiological quality and presence of AMR in foodborne pathogens throughout fresh produce supply chains including the on-farm environment, harvesting, processing and packaging, up to the point of sale. The aim of this study was to determine the microbiological quality and presence of foodborne pathogens (*E. coli*, *Salmonella* spp. and *L. monocytogenes*) in irrigation water and spinach from farm, through processing up to retail. Furthermore, to characterize the isolated *E. coli* phenotypically to compare antibiotic resistance profiles and genotypically (diarrhoeagenic gene screening and enterobacterial repetitive intergenic consensus-polymerase chain reaction [ERIC-PCR] analysis) to determine the similarity and dissemination of *E. coli* within the water–plant–food interface.

MATERIALS AND METHODS

Sampling study areas

Samples were collected from two different commercial spinach production scenarios typically seen in vegetables supply chains in Gauteng Province (Figure 1) as previously described (Richter et al., 2020). River water was used with overhead irrigation and open-field cultivation in the first scenario (Farm A). Depending on the field layout, river water was either used directly or used after storing in a holding dam. For the second spinach production scenario, two farms were selected from various farms supplying a central processing facility for sampling baby spinach grown in tunnels using borehole water for irrigation. A comparison of the farms and their practices is given in Table 1.

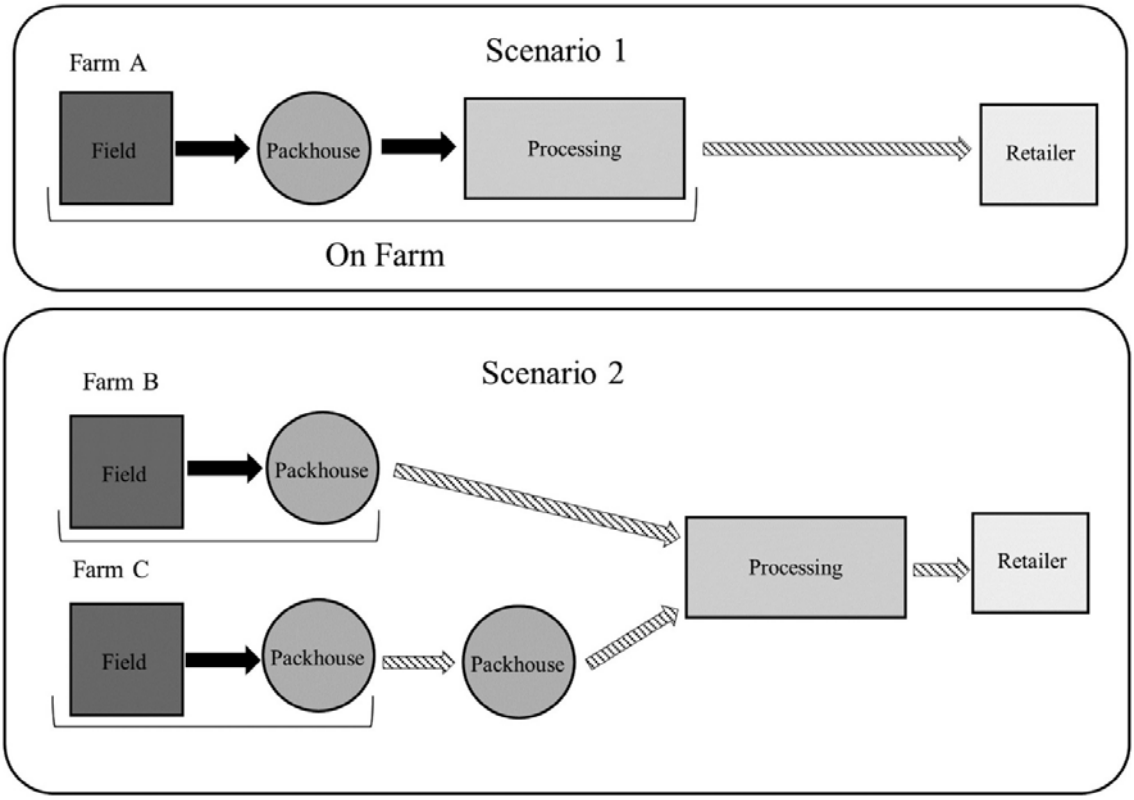

FIGURE 1 Typical spinach production scenarios in Gauteng Province, South Africa. Square brackets show all production practices that occurred on the same farm/premises of each respective scenario. Dashed arrows indicate transportation for processing at a different location and retail of the spinach. In the first scenario, all processing occurred on farm before spinach was transported to commercial retailers or retail distribution centres, while a central processing facility was used in the second scenario were supplier farms with different production practices provided the fresh produce

TABLE 1 Comparison of the processing practices and cultivation of the three spinach farms assessed for this study in 2017

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

Postharvest processing of spinach on Farm A included hand picking and making up of spinach bunches in the field. At the packhouse, spinach bunches were then soaked in a wash bath (containing borehole water) to remove excess soil, labelled and stored in a cold room (4°C, ≤24 h), before transportation to the specific retailers or retailer-distribution centres usually

within 2 days (48 h). Additionally, hand-harvested spinach leaves in crates were also sorted in the packhouse, where the stalks were cut (by hand) and the leaves were put through a cutting machine, chlorine-washed, dried, hand-packed and sealed prior to cold-room storage (4°C, ≤24 h), before transportation to the specific retailers or retailer-distribution centres within a day (24 h).

The baby spinach harvested on Farms B and C were hand sorted along a conveyer belt and packed and weighed in plastic containers in the pack houses on the farm for the unwashed product line, prior to cold-storage and transportation (4°C, ≤24 h) to the processing facility where it was labelled and distributed to the specific retailers. Additionally, baby spinach leaves harvested in crates were cold-stored (4°C, ≤24 h) and transported to the processing facility. At the processing facility, the baby spinach leaves from Farms B and C were cold-stored no longer than 3 days (72 h), chlorine-washed (75–80 ppm active chlorine), packed and sealed before transportation to the specific retailers.

Sample collection

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

Microbiological analysis

Soil

Soil samples were collected from five replicate points during harvest from the spinach production fields. A composite sample of 25 g (5 g from each replicate) was added to 225 ml 3M buffered peptone water (BPW; 3M Food Safety), from which a tenfold dilution series of each soil sample was prepared and plated in duplicate onto *E. coli*/coliform count plates (3M Petrifilm; 3M) for hygiene indicator bacteria enumeration (coliforms, *E. coli*) and on Violet Red Bile Glucose (VRBG; Oxoid) agar plates for Enterobacteriaceae enumeration following incubation for 24 h at 37°C (du Plessis et al., 2015; van Dyk et al., 2016).

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

Water

Water (100 ml and 1 L) samples were collected in triplicate from each sampling point (source, irrigation pivot point and wash water). According to the manufacturer's instructions, the 100 ml water samples were used for enumeration of coliforms and *E. coli* using the most probable number (MPN) with Colilert-18 (IDEXX Laboratories Incorporated) reagents heat-sealed in a Quanti-Tray/2000 (IDEXX). The trays were incubated at 37°C for 24 h and inspected for chromogenic reactions and fluorescence indicating the presence of coliforms and *E. coli* respectively. The results were recorded as log MPN *E. coli* 100 ml^{-1} and log MPN coliforms 100 ml^{-1}. From the 1 L water samples, 1 ml was used to conduct a serial dilution in 9 ml 0.1% BPW, with a 100 µl aliquot from each serial dilution (ranging from 10^{-1} to 10^{-4}) plated in duplicate onto VRBG (Oxoid) agar plates for enumeration of Enterobacteriaceae.

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

Fresh produce

After removal of the spinach stalks, at least three leaves were used to prepare 50 g of composite samples. For the baby spinach, 50 g of composite samples was obtained. Each sample was aseptically cut and placed into a sterile polyethylene strainer stomacher bag (Seward Ltd.) containing 200 ml (3M) BPW in a 1:4 weight to volume ratio. Individual vegetable samples were blended for 5 min at 230 *g* in a Stomacher® 400 Circulator paddle blender (Seward Ltd.). To enumerate hygiene indicator bacteria (coliforms and *E. coli*), a 10-fold dilution series of each BPW sample was made in duplicate, plated onto *E. coli*/coliform count plates and incubated for 24 h at 37°C according to the manufacturer's instructions (3M Petrifilm; 3M, ISO method 4832). Enterobacteriaceae were enumerated by plating 100 µl of the dilution series in duplicate onto VRBG agar plates and incubated for 24 h at 37°C (Oxoid). The remaining BPW samples were incubated for 24 h at 37°C and after enrichment, detection of foodborne pathogens was conducted as described for the soil samples.

Contact surfaces

Transystem™ swabs with Amies medium (Lasec) were used to sample a 25 cm^2 area from crates, tables and conveyer belt surfaces, respectively, in triplicate, according to the standard procedures for environmental swab sampling (Public Health England, 2014). The swab samples were added to 9 ml 3M BPW for enumeration of coliforms/*E. coli* and Enterobacteriaceae as described for the soil samples. The swab samples were subsequently enriched for 24 h at 37°C in BPW. Detection and isolation of *E. coli*, *Salmonella* spp. and *Listeria* spp. were done as described for the soil samples.

All presumptive positive *E. coli*, *Salmonella* spp. and *Listeria monocytogenes* colonies from the soil, water, spinach and contact surface samples were isolated and purified. Isolates were identified using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS; Bruker) to species level as described by Standing et al. (2013) and AOAC-OMA#2017.09. Briefly, the purified presumptive positive colonies were regrown in 9 ml tryptone soy broth (TSB; MERCK) and incubated overnight at 37°C. Subsequently, isolates

(10 µl) were streaked out on Nutrient Agar (MERCK) and the plates were incubated overnight at 37°C and subjected to the MALDI Biotyper protocol (Bruker) (Standing et al., 2013). All strains were tested in duplicate.

Antimicrobial susceptibility testing

The *E. coli* isolates ($n = 80$) from the different spinach production scenarios were further tested for AMR against seven antibiotic classes. The Kirby-Bauer disk diffusion technique was used to determine the resistance patterns of the isolates (Clinical Laboratory Standard Institute [CLSI], 2017). Briefly, each isolate was cultured in 9 ml TSB and incubated for 24 h at 37°C. Of each TSB sample, 100 µl was subsequently inoculated into 9 ml brain heart infusion broth (MERCK) and incubated for 24 h at 37°C. A 120 µl bacterial suspension was then plated onto Mueller-Hinton agar plates (MERCK) and screened for resistance against 11 antibiotics belonging to seven classes. (Mast Diagnostics, supplied by Davies Diagnostics) using the Disk Master Disc dispenser (Mast Diagnostics), and incubated for 16–18 h at 37°C. Antibiotics screened for included ampicillin—10 µg, amoxicillin-clavulanic acid—20 µg/10 µg, amoxicillin—10 µg, trimethoprim-sulfamethoxazole/cotrimoxazole—1.25 µg/23.75 µg, cefoxitin—30 µg, cefepime—30 µg, imipenem—10 µg, neomycin—10 µg, tetracycline—30 µg, gentamycin—10 µg and chloramphenicol—30 µg (Mast Diagnostics) (CLSI, 2017). Breakpoints were then compared to (CLSI, 2017) and isolates resistant to three or more antimicrobial classes were regarded as multidrug resistant. *E. coli* ATCC 25922 was included as a control (CLSI, 2017).

Molecular characterization of diarrhoeagenic *E. coli*

The presence of different diarrheoagenic *E. coli* virulence genes for enterotoxigenic *E. coli* (ETEC) (*lt* and *st* genes), enteropathogenic *E. coli* (EPEC) (*bfpA* and *eaeA* genes), enteroaggregative *E. coli* (Eagg) (*eagg* gene), enterohaemorrhagic *E. coli* (EHEC) (*eaeA*, *stx1* and *stx2* genes) and enteroinvasive *E. coli* (EIEC) (*ipaH* gene) were analysed by PCR and sequencing, with the *mdh* gene used as an internal control in all reactions (Table S1) (Omar & Barnard, 2010). *E. coli* control strains for the PCR reactions included DSM 10973 and DSM 27503 (ETEC); DSM 8703 and DSM 8710 (EPEC); DSM 27502 (Eagg); DSM 9028 and DSM 9034 (EIEC); *E. coli* O157:H7 (ATCC 35150) (EHEC) and ATCC 25922 (negative control).

Single colonies of each *E. coli* isolate were cultured aerobically under shaking conditions at 200 *g* in TSB (MERCK) for 24 h at 30°C. The cells were pelleted by centrifugation (12,500 *g* for 10 min), DNA was extracted using the Quick-gDNA Mini-Prep kit (Zymo Research) and the DNA concentration was determined using the Qubit dsDNA Broad Range Assay and a Qubit 2.0 fluorometer (Life Technologies). PCR was performed using 1× DreamTaq Green PCR Master Mix (ThermoFisher Scientific), with specific primers, and thermocycling conditions for each of the genes as described in Table S1.

Genomic fingerprinting of *E. coli* by repetitive PCR

The same *E. coli* isolates analysed for antimicrobial susceptibility and virulence genes were used to conduct repetitive PCR through the generation of ERIC-PCR fingerprints from each individual spinach production scenario. PCR was performed using 1× DreamTaq Green PCR Master Mix (ThermoFisher Scientific), 80–100 ng template DNA and 4 µM of each primer in a total reaction volume of 25 µl. The forward and reverse primer sequences used to generate the DNA fingerprints were 5′-ATGTAAGCTCCTGGGGATTCAC-3′ and 5′-

AAGTAAGTGACTGGGTGAGCG-3′ respectively (Soni et al., 2014). The PCR conditions were: 95°C for 4 min, followed by 30 cycles of 94°C for 30 s, 40°C for 1 min and 72°C for 8 min, with a final elongation step at 72°C for 15 min. The PCR amplicons were visualized in a 2% agarose gel and band patterns were analysed and compared using Bionumerics 7.6 fingerprint analyst software (Applied Maths). The percent similarities of digitized bands were calculated using the Pearson's correlation coefficient and the unweighted pair group method with arithmetic mean, and complete linkage algorithms were used to derive a dendrogram.

Statistical analysis

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

RESULTS

Microbiological quality analysis

The *E. coli*, coliforms and Enterobacteriaceae levels in the analysed irrigation water, wash water and spinach from the farm, through processing and at the retailer are shown in Figures 2–4, while fluctuations of counts within each respective chain and results of statistical analysis are shown in Tables S2–S9.

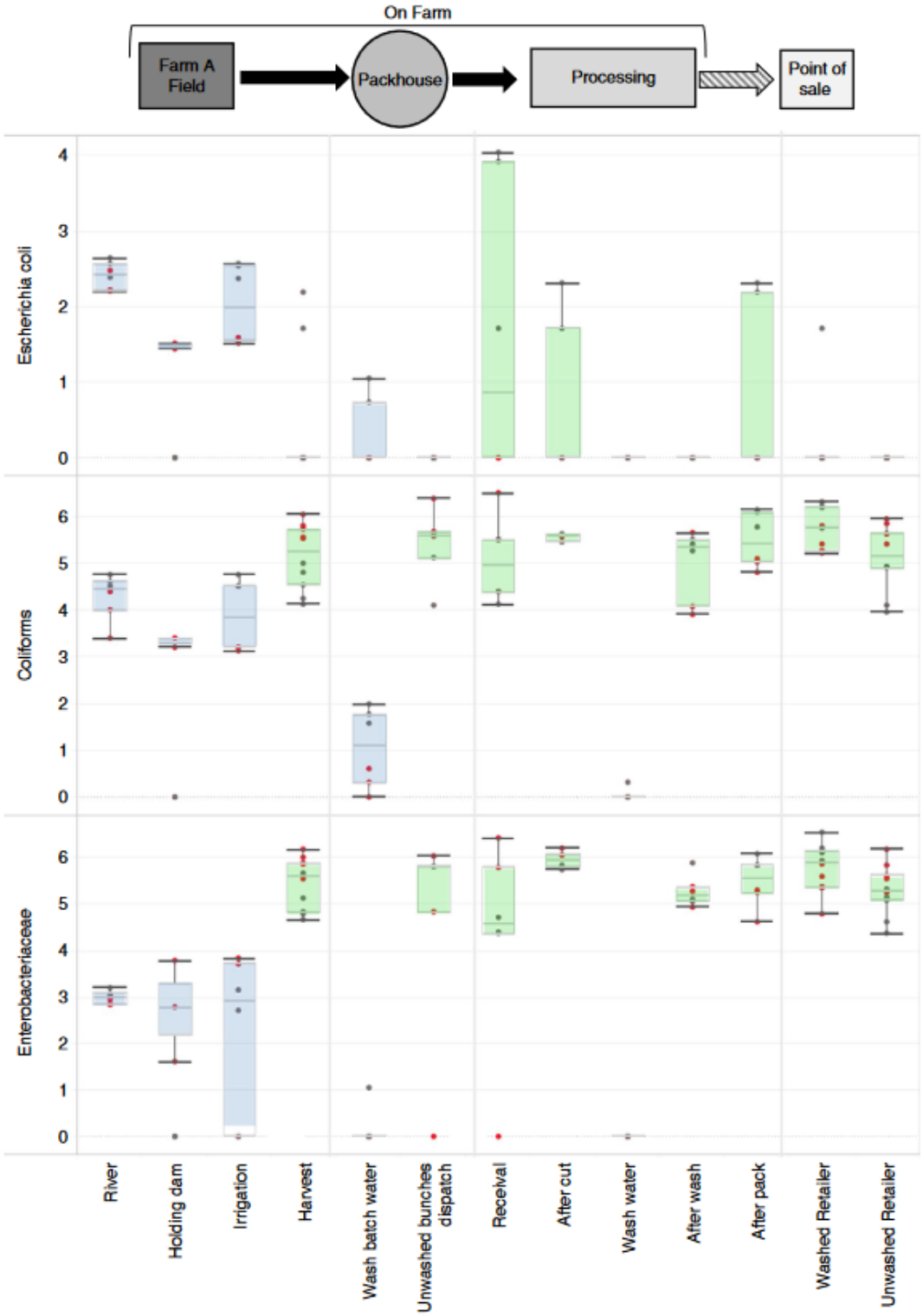

FIGURE 2 Indicator bacteria levels from water (log MPN 100 ml^{-1}) and spinach (log CFU g^{-1}) from farm to retail in a spinach production system using river water for irrigation (: spinach (log CFU g^{-1}); : water (log MPN 100 ml^{-1}). ■: Trip 2; ■: Trip1). CFU, colony forming unit; MPN, most probable number

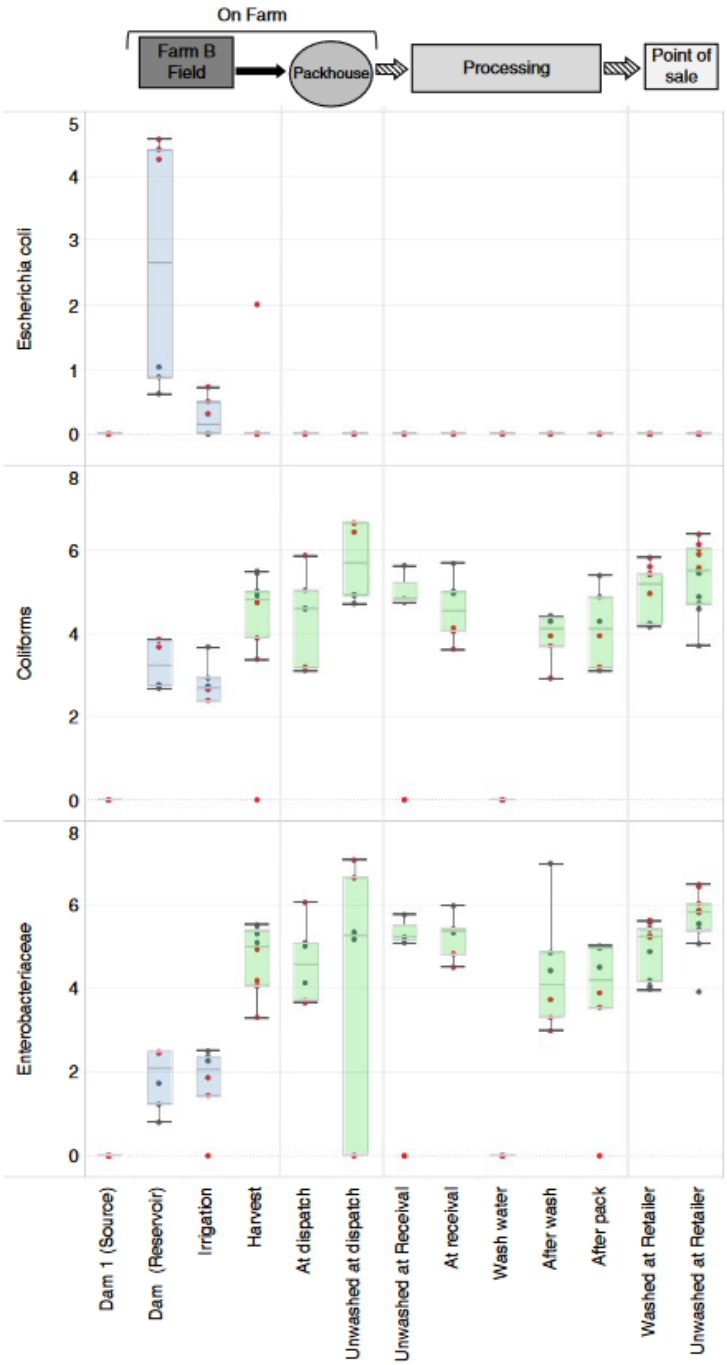

FIGURE 3 Indicator bacteria levels from water (log MPN 100 ml^{-1}) and spinach (log CFU g^{-1}) from farm to retail in a spinach production system using borehole water for irrigation and produce were processed at a centralized processing facility.(: spinach (log CFU g^{-1}); : water (log MPN 100 ml^{-1}). : Trip 2; : Trip1). CFU, colony forming unit; MPN, most probable number

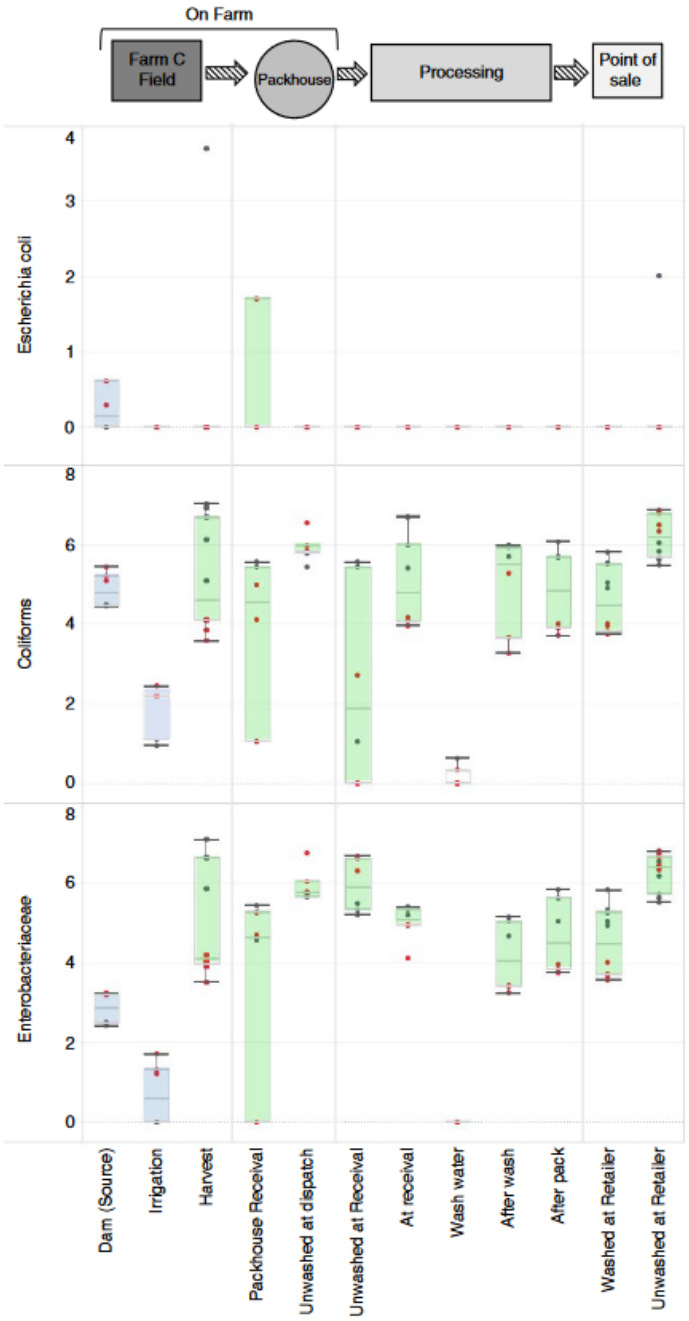

FIGURE 4 Indicator bacteria levels from water (log MPN 100 ml^{-1}) and spinach (log CFU g^{-1}) from farm to retail in a spinach production system using borehole water for irrigation and produce were processed at a centralized processing facility. (: spinach (log CFU g^{-1}); : water (log MPN 100 ml^{-1}). ■: Trip 2; ■: Trip1). CFU, colony forming unit; MPN, most probable number

In the first production scenario, the *E. coli* levels in river water ranged from 2.20 to 2.64 log MPN 100 ml^{-1}, in the holding dam water from 1.43 to 1.50 log MPN 100 ml^{-1} and in the irrigation pivot point water from 1.50 to 2.56 log MPN 100 ml^{-1} (Figure 2). These *E. coli* levels were higher than the national regulation limits for vegetable and crop irrigation water (<1000 *E. coli* 100 ml^{-1}) (Department of Water Affairs & Forestry [DWAF], 1996). The river water *E. coli* levels during Trip 1 were significantly higher than that of the holding dam and irrigation pivot point water samples (p = 0.0257) (Table S2). During Trip 2, river was directly used for irrigation, subsequently, the *E. coli* levels in the irrigation pivot point and river water samples were not significantly different (p = 0.0257) (Table S2). The coliform levels of river, holding dam and irrigation pivot point water samples from Farm A ranged from 3.38 to 4.76 log MPN 100 ml^{-1}, from 3.19 to 3.38 log MPN 100 ml^{-1} and from 3.11 to 4.76 log MPN 100 ml^{-1} respectively. Similar to the *E. coli* counts, differences were observed in the coliform levels, with the

counts from the river water during Trip 1 being higher than the holding dam and irrigation pivot point water samples during the same trip (p = 0.0077) (Table S2). Enterobacteriaceae counts in river water from Farm A ranged from 2.84 to 3.20 log colony forming unit (CFU) ml^{-1}, while the holding dam and irrigation pivot point counts ranged from 1.61 to 3.78 log CFU ml^{-1} and from 0.00 to 3.83 log CFU ml^{-1} respectively (Figure 2).

The *E. coli* levels on spinach from Farm A ranged from 0.00 to 4.03 log CFU g^{-1}. The *E. coli* (trip × source) count interactions from spinach were significantly different (p = 0.0012) (Table S3). No *E. coli* was enumerated from any of the spinach samples during Trip 1. Where river water was used directly for overhead irrigation during Trip 2, *E. coli* were enumerated from harvested spinach, the unwashed spinach bunches as well as spinach at receival in the packhouse, spinach after cut, after wash, after pack and the retailed samples of the washed spinach product line (Figure 2). The *E. coli* levels during Trip 2 on spinach at receival were significantly higher (p = 0.0012) than spinach at harvest, after cut and after pack, with all other samples having significantly lower *E. coli* levels (p = 0.0012) (Table S3). The coliform and Enterobacteriaceae levels on spinach from Farm A ranged from 3.90 to 6.50 log CFU g^{-1} and from 0.00 to 6.52 log CFU g^{-1} respectively.

For the second production scenario, *E. coli* counts in borehole water used for irrigation on Farm B were 0.00 log MPN 100 ml^{-1} (Figure 3). The reservoir dam water (Trip 1 and Trip 2) and irrigation pivot point (Trip 1) *E. coli* counts ranged between 0.61 and 4.56 log MPN 100 ml^{-1} and between 0.00 and 0.72 log MPN 100 ml^{-1}, respectively, and were significantly higher (p < 0.0001) than that of the borehole source water (Figure 3; Table S5). Moreover, the *E. coli* levels of the reservoir dam water sampled during Trip 2 were unacceptable according to the national regulation for irrigation water (DWAF, 1996). However, the *E. coli* levels measured during the same trip at the irrigation pivot point in the field were significantly lower and with acceptable levels according to the guidelines (Table S5). Similarly, the coliform and Enterobacteriaceae counts from the water samples were significantly different (p < 0.0001) (Table S5). The coliform counts of the borehole water were 0.00 log MPN 100 ml^{-1}, while the coliform counts from the reservoir dam and irrigation pivot point water samples ranged between 2.65 and 3.84 log MPN 100 ml^{-1} and between 2.35 and 3.64 log MPN 100 ml^{-1} respectively (Figure 3). Similar results were obtained for the Enterobacteriaceae counts of the borehole, reservoir and irrigation pivot point water from Farm B (Figure 3).

The *E. coli* counts of the Farm B spinach samples from harvest up to the retailer ranged between 0.00 and 2.00 log CFU g^{-1} (Figure 3), and were not significantly different (p = 0.7069) (Table S5). Coliform and Enterobacteriaceae counts on spinach from Farm B ranged between 0.00 and 6.65 log CFU g^{-1} and between 0.00 and 7.05 log CFU g^{-1}, respectively (Figure 3), with significant differences observed in the trip x source interactions (Table S6).

On Farm C, *E. coli* was enumerated in low levels during Trip 1 from the source dam water (borehole) only, with counts ranging between 0.00 and 0.61 log MPN 100 ml^{-1}. The *E. coli* levels from the water samples were significantly different (p = 0.0014) (Table S7), with counts in water from the source dam being significantly higher during Trip 1. Coliform counts in the irrigation water from Farm C ranged between 4.44 and 5.44 log MPN 100 ml^{-1} and between 0.93 and 2.44 log MPN 100 ml^{-1} in the borehole source and irrigation pivot point water samples respectively. The Enterobacteriaceae levels ranged between 2.41 and 3.23 log CFU ml^{-1} and between 0.00 and 1.71 log CFU 100 ml^{-1} in the borehole source and irrigation pivot water samples respectively (Figure 4). Similar to the *E. coli* counts on spinach from Farm B, the *E. coli* counts on spinach from Farm C ranged between 0.00 and 3.70 log CFU g^{-1} (Figure 4),

with no significant difference ($p = 0.6166$) in *E. coli* levels on spinach from harvest up to retail (Table S8). The coliform counts on spinach from Farm C ranged between 1.04 and 7.01 log CFU g^{-1} (Figure 4) and had significant differences ($p < 0.0001$) (Table S8). Similarly, the Enterobacteriaceae levels on spinach ranged from 0.00 to 7.07 log CFU g^{-1} (Figure 4), with significant differences in the trip × source interactions ($p < 0.0001$) (Table S8).

The composite soil samples of the three farms had similar mean Enterobacteriaceae and coliform counts, ranging between 3.29 and 5.22 log CFU g^{-1} and between 3.05 and 5.19 log CFU g^{-1}, respectively, with no *E. coli* enumerated from soil on any of the farms (Table S10).

Detection of indicator organisms and potential foodborne pathogens

Overall, 65/288 samples (22.57%) contained *E. coli* after enrichment. A higher number of *E. coli* isolates were recovered from the second production scenario after enrichment, yet the enumerated *E. coli* levels were higher from the first production scenario. *E. coli* isolates (n = 80) were recovered from the two spinach production scenarios. This included 35 isolates from the first production scenario from soil (n = 1), water (n = 13), fresh produce (n = 14) and contact surfaces (n = 7), while the 45 *E. coli* isolates recovered from the second production scenario were from water (n = 29) and fresh produce (n = 16). Only one *E. coli* isolate from the holding dam water in the first production scenario, was positive for the *stx2* virulence gene, while none of the other diarrhoeagenic virulence genes tested for were detected. *Salmonella* spp. isolates (n = 11) were recovered from river (n = 4), holding dam (n = 1) and irrigation pivot point (n = 4) water samples from the first production scenario. No *Listeria* spp. were isolated from any of the samples.

Phenotypic AMR profiling of *E. coli* isolates

Of the 80 *E. coli* isolates recovered, 95% were resistant against at least one antibiotic. This included resistance to aminoglycosides (73.42%), cephalosporins (50.62%), penicillins (44.30%), tetracyline (37.98%), sulphonamides (21.52%), chloramphenicol (15.19%) and carbapenems (5.06%). Overall, a greater percentage of resistance phenotypes were from water *E. coli* isolates (52.50%), followed by isolates from spinach (37.50%) and contact surfaces (10%) (Figures 5 and 6) In total, 35/80 (43.75%) of the isolates were multidrug resistant; 26.30% from production scenario one, and 17.50% from the second production scenario, where borehole water was used for irrigation (Table 2). The multidrug-resistant *E. coli* isolates predominantly showed, within the β-lactam group, resistance to penicillins (66.3%), followed by fourth-generation cephalosporins (61.3%) and carbapenems (11.3%). Multidrug-resistant phenotypes predominantly included resistance profiles of β-lactams combined with aminoglycosides, followed by β-lactams combined with tetracyclines, sulfonomides and chloramphenicol respectively (Table 2).

TABLE 2 Summary of the number of antimicrobials, most frequent resistance patterns, number and type of antibiotic classes to which generic *Escherichia coli* isolates from different spinach production scenarios were resistant

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

(Continues)

TABLE 2 (Continued)

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

TABLE 2 (Continued)

No of antimicrobials to which isolates were resistant	No of isolates ($n = 79$)	No of isolates per production scenario		No of isolates with specific pattern	Most frequent pattern	No of antibiotic classes to which isolates were resistant	Antibiotic class(es)
		Production scenario 1	Production scenario 2				
7	9	1		1	AP10C–AUG30C–A10C–FOX30C–CPM30C–T30C–NE10C	4	Penicillins, Cephalosporins, Tetracyclines, Aminoglycosides
		5		5	AP10C–AUG30C–A10C–TS25C–T30C–NE10C–C30C	5	Penicillins, Sulphonamides, Tetracyclines, Aminoglycosides, Chloramphenicol
		1		1	AP10C–AUG30C–A10C–CPM30C–TS25C–T30C–NE10C	5	Penicillins, Cephalosporins, Sulphonamides, Tetracyclines, Aminoglycosides
			1	1	AP10C–A10C–CPM30C–TS25C–GM10C–T30C–NE10C	5	Penicillins, Cephalosporins, Sulphonamides, Aminoglycosides, Tetracyclines
			1	1	AP10C–AUG30C–A10C–CPM30C–TS25C–T30C–C30C	5	Penicillins, Cephalosporins, Sulphonamides, Tetracyclines, Chloramphenicol
8	1		1	1	AP10C–AUG30C–A10C–FOX30C–CPM30C–TS25C–GM10C–NE10C	4	Penicillins, Cephalosporins, Sulphonamides, Aminoglycosides
9	2	1		1	AP10C–AUG30C–A10C–CPM30C–TS25C–GM10C–T30C–NE10C–C30C	6	Penicillins, Cephalosporins, Sulphonamides, Aminoglycosides, Tetracyclines, Chloramphenicol
		1		1	AP10C–AUG30C–A10C–CPM30C–TS25C–IMI10C–T30C–NE10C–C30C	7	Penicillins, Cephalosporins, Sulphonamides, Carbapenems, Tetracyclines, Aminoglycosides, Chloramphenicol

Abbreviations: AP10C, ampicillin; AUG30C, amoxycillin-clavulanic acid; A10C, amoxycillin; C10C, chloramphenicol; CPM30C, cefepime; FOX30C, cefoxitin; GM10C, gentamycin; IMI10C, imipenem; NE10C, neomycin; TS25C, trimethoprimsulfamethoxazole/cotrimoxazole; T30C, tetracycline

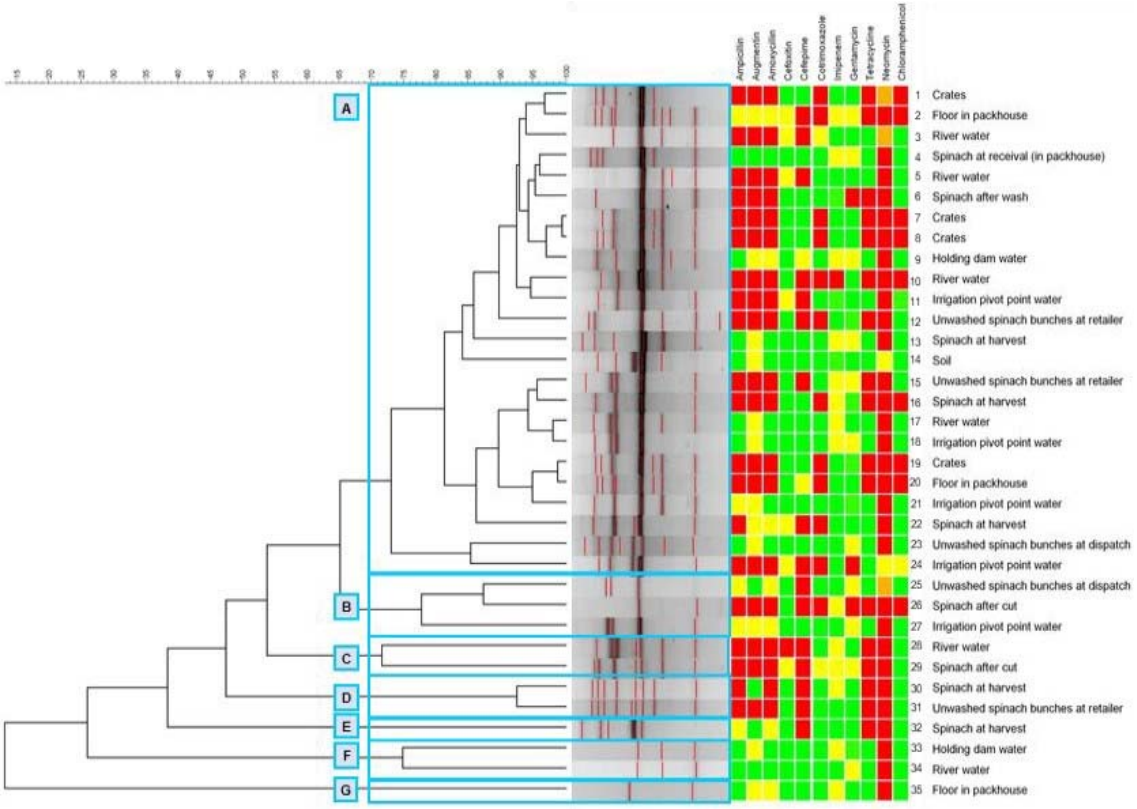

FIGURE 5 Dendrogram showing the genetic relatedness of *Escherichia coli* isolates from irrigation water sources (river, holding dam and irrigation pivot point), soil, spinach (at harvest, throughout processing and at retail) and contact surfaces throughout spinach production at a 70% similarity cut-off in clusters A to G. (■: Resistant; ■: Intermediate; ■: Susceptible)

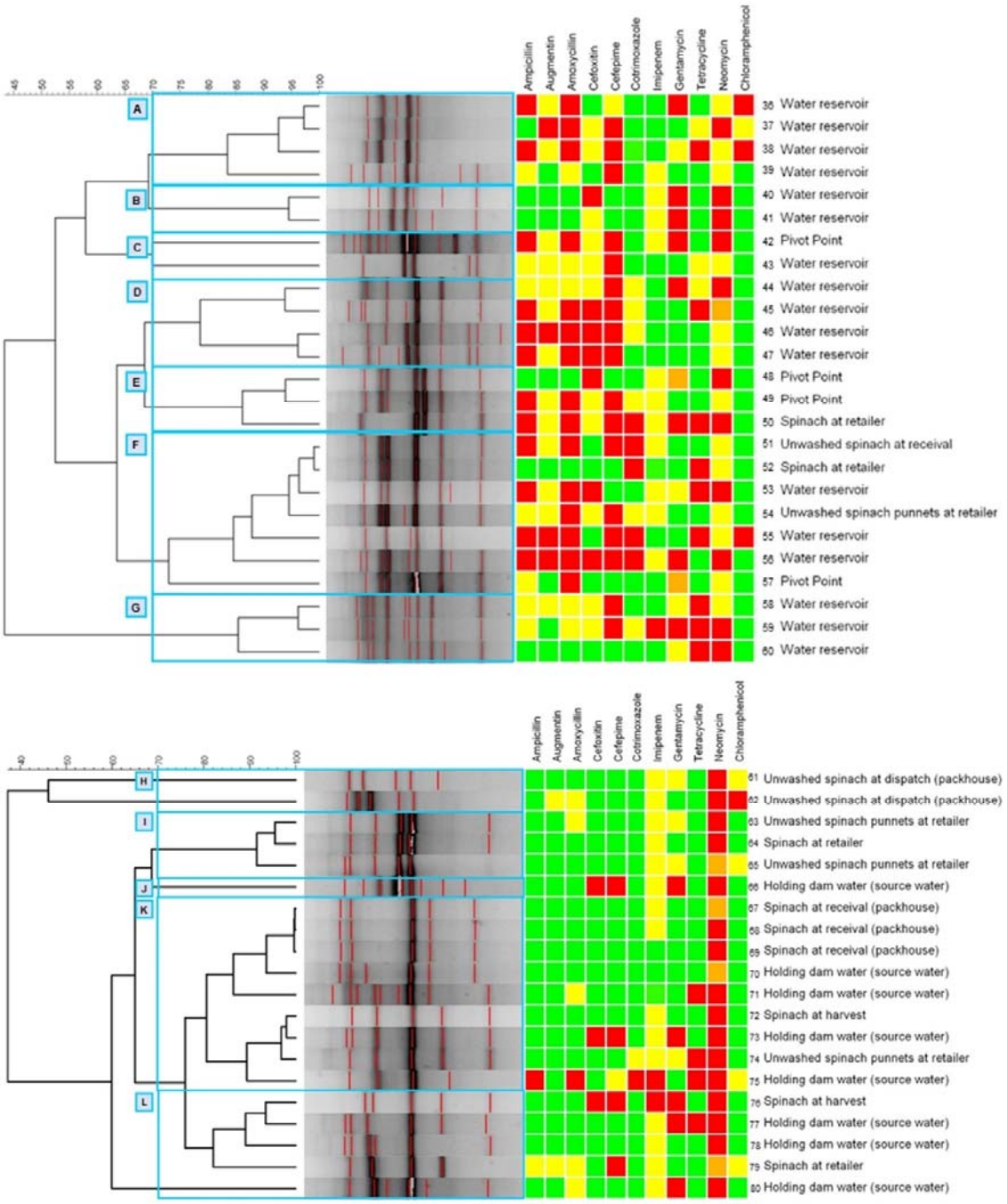

FIGURE 6 Dendrogram showing the genetic relatedness of *Escherichia coli* isolates from irrigation water sources (borehole water sources) and spinach (at harvest, throughout processing and at retail) from two farms supplying spinach to a central processing facility at a 70% similarity cut-off in clusters A to L. (■: Resistant; ■: Intermediate; ■: Susceptible)

ERIC–PCR cluster analysis and AMR profiles of *E. coli* isolates

At a 70% similarity cut-off, cluster analysis of ERIC-PCR DNA fingerprints generated seven distinct *E. coli* profiles for the 35 isolates from the first production scenario (Figure 5A–G). The largest cluster (Cluster A) included *E. coli* isolates ($n = 24$) from water, soil, spinach from farm to retail, as well as contact surfaces through processing. Several irrigation water and contact surface samples, as well as spinach at different points throughout production clustered together in cluster A with ≥94.0% similarity values. Cluster B included isolates from spinach at different points in the packhouse and irrigation water with similarity values of 78.0%. Similarly, cluster C included an *E. coli* isolate from spinach after the cut that was 72.0% similar to a river water isolate. Cluster D was composed of two *E. coli* isolates from spinach (at harvest and at retail) at similarity values >90.0%, while in cluster F, two *E. coli* isolates from the river and holding dam water clustered together at 75.0% similarity. Cluster G consisted of a single *E. coli* isolate from the floor swab samples. The *E. coli* ERIC-PCR DNA fingerprints in the second production scenario generated 12 distinct clusters. This included seven clusters in the supply chain from the first supplier, Farm B (Figure 6A–G) and five clusters in the supply chain from the second supplier, Farm C (Figure 6H–L). Cluster E was composed of three *E. coli* isolates from the irrigation pivot point and spinach at retailer, with 86.0% similarity values. In cluster F, several *E. coli* isolates from the water reservoir, spinach at receival in the packhouse as well as washed and unwashed retail spinach clustered together at similarity values ranging from 73.0% to 99.0%. In cluster I, three *E. coli* isolates from the washed and unwashed spinach product lines at the retailer clustered together with 92.0% similarity. Clusters K consisted of nine *E. coli* isolates, including three spinach at receival isolates and one holding dam isolate with 94.0% similarity. Furthermore, *E. coli* isolates from spinach at harvest, holding dam (source water) and the unwashed spinach at retailer had 98.0% similarity. The five isolates in cluster L included three *E. coli* isolates from spinach at harvest, and holding dam (source) water with 90.0% similarity.

DISCUSSION

To the authors knowledge, this is the first study in SA where complete spinach production systems with different irrigation water sources from the farm, throughout processing and up to retail, were investigated for the presence of multidrug-resistant foodborne pathogens and quality indicator organisms. As water is central in fresh produce production and processing, and applied in large volumes, it is crucial that the microbiological quality is acceptable (Makinde et al., 2020). Inconsistencies of irrigation water sources, guidelines and regulations, however, result in complex assessment and mitigation strategies globally. When spinach was irrigated directly with river water via overhead irrigation in this study, *E. coli* was enumerated from the irrigation water, spinach, contact surface and wash water samples throughout the supply chain. The average river water *E. coli* levels (2.4 log MPN 100 ml^{-1}) were similar to the results reported for river water used for overhead irrigation of commercially produced leafy greens in a previous study in Gauteng Province (2.9 log MPN 100 ml^{-1}) (Jongman & Korsten, 2016). In contrast, *E. coli* was not enumerated from the river water used to irrigate produce in KwaZulu Natal, South Africa (Mdluli et al., 2013). According to the SA DWAF guidelines of <1000 *E. coli* 100 ml^{-1} for irrigation water (DWAF, 1996), the river water *E. coli* levels in the current study would have been satisfactory. This is also in agreement with the World Health Organisation (WHO) recommendation of <1000 CFU *E. coli* 100 ml^{-1} in irrigation water used for minimally processed fresh produce (WHO, 2006). However, the river water *E. coli* levels exceeded the Canadian standards' acceptable limit of <100 *E. coli* 100 ml^{-1} for irrigation water used for produce to be consumed raw (Canadian Council of Ministers of the Environment

[CCME], 2003) and the European Union (EU) limit of 100 *E. coli* 100 ml^{-1} in irrigation water used for fresh fruit and vegetables (likely to be eaten uncooked) with the edible portion in direct contact of the irrigation water (European Commission [EC], 2017). Additionally, fresh produce industries such as the Leafy Greens Marketing Agreement (LGMA) in the U.S. has commodity-specific guidelines for irrigation water used for production and harvest of leafy greens (FDA, 2021). The guidelines are based on the U.S. Food Safety Modernisation Act (FSMA) with a strong food safety focus shifting from responding to preventing foodborne illness (FDA, 2021). The LGMA and produce safety rule of the FSMA propose a water microbiological quality standard of average generic *E. coli* levels <126 MPN 100 ml^{-1} for multiple samples of irrigation water used in leafy green production (Haymaker et al., 2019). The river water *E. coli* levels from the current study would not have been compliant according to the FSMA irrigation water guidelines.

Where borehole water was used for irrigation, the source water *E. coli* levels from the first supplier farm (Farm B) met the current SA and WHO irrigation water standards of <1000 *E. coli* 100 ml^{-1} (DWAF, 1996; WHO, 2006). *E. coli* levels in the holding dam water did not meet this requirement, reiterating that water quality may affect the microbiological quality of irrigated produce. The *E. coli* levels in the source water from the second supplier farm in production scenario two was acceptable according to the SA national regulation limits (DWAF, 1996) as well as the EU, FSMA and Canadian standards' acceptable limit (CCME, 2003; European Commission, 2017; FDA, 2021). Internationally, guidelines and regulations for agricultural water quality vary by country/region with different acceptable *E. coli* limits stipulated based on the risk of types of agricultural water systems and specific uses within production and processing (Banach & van der Fels-Klerx, 2020). Aligning national water microbiological quality guidelines for the production and processing of fresh produce with international regulations and standards is of particular importance when considering the export markets and increasing globalized production. However, the availability of water resources, which is under increasing pressure, varies between countries (Uyttendaele et al., 2015), adding to the complexity of establishing a global microbiological quality standard for fresh produce irrigation water. The wash water during processing from the current study had acceptable *E. coli* levels according to international guidelines of *E. coli* <100 CFU ml^{-1} in prewash water to remove soil and debris (Australia and New Zealand Fresh Produce Safety Centre) or water used for first washing of ready-to eat products (EU), and *E. coli* <1 CFU 100 ml^{-1} in water for the final wash step of produce that may be eaten uncooked (European Commission, 2017; Fresh Produce Safety Centre Australia & New Zealand [FPSC A-NZ], 2019). In addition to water quality in fresh produce production, agricultural and harvesting practices from farm to retail may also influence the microbiological quality and safety of fresh produce (Machado-Moreira et al., 2019).

The microbiological characteristics of raw fruit and vegetables are one of the most important properties related to safe fresh produce consumption (Faour-Klingbeil et al., 2016; Schuh et al., 2020). Internationally, no consensus exists regarding the microbiological standards that apply to ready-to-eat (RTE)/minimally processed vegetables (Health Protection Agency, 2009; Food Safety Authority of Ireland [FSAI], 2016; FPSC A-NZ, 2019). A number of countries do suggest exclusion of coliform counts, as high levels are expected due to the natural occurrence (Centre for Food Safety [CFS], 2014; Health Canada, 2010; New South Wales Food Authority, 2007). In SA, the Department of Health (DoH) guidelines stipulated that coliform levels of <2.3 log CFU g^{-1} were acceptable on fresh vegetables (DoH, 2000), however, these guidelines are currently under revision. Coliforms were enumerated from 98% of the spinach samples in the current study with levels that exceeded 2.3 log CFU g^{-1}, similar to other South African

studies that reported coliform levels >2.3 log CFU g^{-1} on retailed leafy green vegetables (du Plessis et al., 2017; Richter et al., 2021). Globally, high coliform levels in retailed leafy greens have also been reported (Cerna-Cortes et al., 2015; Korir et al., 2016; Maffei et al., 2016).

In contrast to the coliforms, *E. coli* was only enumerated from 8.33% of the spinach samples, thus, 91.6% of the spinach samples had acceptable *E. coli* levels according to the previous DoH *E. coli* guidelines of 0 CFU g^{-1} (DoH, 2000). The EU guidelines for *E. coli* limits on RTE precut fruit and vegetables state that levels <100 CFU g^{-1} are satisfactory, *E. coli* levels between 10^2 and 10^3 CFU g^{-1} are borderline and samples with *E. coli* >10^3 CFU g^{-1} are unsatisfactory (European Commission, 2007). Interestingly, the spinach samples where *E. coli* was enumerated in the current study, included predominantly spinach samples from the first production scenario, during Trip 2, where river water was directly applied for irrigation. The spinach *E. coli* counts throughout the chain in this scenario ranged between 1.71 and 4.03 log CFU g^{-1}, and the washed samples after pack and at the point of sale would have been borderline according to the EU guidelines for *E. coli* limits on RTE precut fruit and vegetables. Additionally, *E. coli* was enumerated from unwashed retailed spinach samples from the second production scenario where borehole water was used for irrigation with levels that would also have been borderline (between 10^2 and 10^3 CFU g^{-1}) according to these guidelines (European Commission, 2007).

The natural occurrence of Enterobacteriaceae on spinach at various stages of production and processing, regardless of the source of irrigation water, was expected (Al-Kharousi et al., 2018; Berg et al., 2014; Leff & Fierer, 2013). In the current study, Enterobacteriaceae levels on packed, washed retail spinach samples ranged between 3.56 and 6.52 log CFU g^{-1} and on unwashed retail spinach samples between 3.92 and 6.78 log CFU g^{-1}. Similar Enterobacteriaceae levels were reported on minimally processed and unprocessed vegetables in Italy, suggesting that the microbial flora can be primarily attributed to a natural environmental source (Al-Kharousi et al., 2018; Cardamone et al., 2015). However, higher Enterobacteriaceae loads could also represent higher loads of potential pathogens such as *E. coli* and *Salmonella* spp. and opportunistic pathogens including *Klebsiella pneumoniae* and *Enterobacter* species (Kilonzo-Nthenge et al., 2018). Furthermore, resistance gene dissemination among micro-organisms is not restricted to geographical zones, therefore, a global collaborative multisectoral approach to detect, prevent and respond is vital.

After enrichment, generic *E. coli* was isolated from 40.30% and 14.60% of water and spinach samples respectively. This was lower than the 84.80% and 38.30% generic *E. coli* prevalence in irrigation water and lettuce samples previously reported in Brazil (Decol et al., 2017). Similar to du Plessis et al. (2015) and Decol et al. (2017), more irrigation water samples in the current study were contaminated with *E. coli* than fresh produce samples. Additionally, only one water *E. coli* isolate was positive for the *stx2* virulence gene. This corresponds to previous South African studies where a low incidence of virulence genes in *E. coli* from retailed fresh produce was seen (du Plessis et al., 2017; Jongman & Korsten, 2016a; Richter et al., 2021). In the current study, no *Salmonella* spp. were isolated from any of the spinach samples, however, the river irrigation water samples from the first production scenario were positive for *Salmonella* spp. Similarly, Castro-Ibáñez et al. (2015) have reported low prevalence of *Salmonella* spp. in irrigation water samples of commercially produced spinach, with no isolates from the spinach samples. Selected *Salmonella* spp. isolates from the current study were screened for AMR (data not shown), and the isolates with extended-spectrum β-lactamase resistance profiles have previously been reported (Richter et al., 2020). Furthermore, no spinach samples from the current study harboured *L. monocytogenes*, which corresponds to a

previous study of retailed fresh produce sold formally and informally (Richter et al., 2021). However, previous studies have confirmed that spinach supports the growth of *L. monocytogenes*, with the retailed product not showing any obvious deterioration (Culliney et al., 2020). This poses a serious health risk to consumers, making surveillance of *L. monocytogenes* together with potential pathogenic Enterobacteriaceae in food supply crucial, as leafy greens have previously been implicated in listeriosis outbreaks, including a multistate outbreak in the U.S. (Self et al., 2019). Although *Salmonella* spp. were only detected in 3% of the samples in the current study, the presence of potential foodborne pathogens, as well as antibiotic-resistant commensal bacteria highlights irrigation water as a potential risk factor for introduction of resistance genes and pathogens in leafy green primary production, which agrees with previous studies (Castro-Ibáñez et al., 2015; Vital et al., 2018).

Knowledge of bacterial AMR patterns, is crucial for the reduction of the number of treatment failures if a foodborne disease outbreak does occur (Kim et al., 2019). Previously, commensal bacteria have been reported to harbour clinically significant AMR genes as well as mobile genetic elements, which is concerning when considering resistance gene transfer to opportunistic and pathogenic bacteria (Al-Kharousi et al., 2018). In this study, 95% *E. coli* isolates were resistant to at least one antibiotic with 43.75% being multidrug resistant. *E. coli* isolates from both irrigation water and spinach in the current study were resistant to antibiotics that are traditionally first-line drug treatment options for gastrointestinal infections (tetracycline, ampicillin and cotrimoxazole) (Alanazi et al., ; Kim et al., 2019). More antibiotic-resistant *E. coli* isolates were detected from irrigation water (52.5%) than from spinach (37.5%) in the current study, which is similar to antibiotic-resistant *E. coli* isolates reported in irrigation water and harvested spinach by Vital et al. (2018). The highest resistance in irrigation water *E. coli* isolates from the current study was against aminoglycosides (35.0%), followed by cephalosporins (28.8%), penicillins (23.8%) and tetracycline (15.0%). In contrast, Vital et al. (2018) reported the highest resistance in *E. coli* isolates from irrigation water in the Philippines against tetracycline (45.6%) and ampicillin (34%). The results from the current study, similar to AMR reported in *E. coli* from irrigation water and harvested leafy greens in other studies (Summerlin et al., 2021; Vital et al., 2018), indicates the need for expanded AMR surveillance systems in the water–plant–food interface, that can be integrated with AMR surveillance systems in other sectors. Currently, AMR in foods of plant origin is not well documented, especially in low- and middle-income countries (Food & Agriculture Organization [FAO], 2018). However, selected studies have previously shown the potential of linking *E. coli* as AMR indicator bacteria between irrigation water and fresh produce, through phenotypic AMR analysis and DNA fingerprinting (du Plessis et al., 2015; Njage & Buys, 2014).

The ERIC-PCR profiles in the current study showed high similarity values (>90.0%) for irrigation water and spinach *E. coli* isolates at different points of production, processing or retail of each of the respective supply chains. Previous studies have reported the transfer of potential pathogenic enteric bacteria onto produce via irrigation with polluted water (du Plessis et al., 2015; Ijabadeniyi, 2012). For example du Plessis et al. (2015) highlighted the link between irrigation water quality and microbiological quality of onions, while Jongman and Korsten (2016a) showed a link between *E. coli* isolates from different leafy green vegetables and the associated irrigation water. Interestingly, cluster analysis within each spinach supply chain in the current study (regardless of the water source and overall microbiological quality of the irrigation water) showed irrigation water *E. coli* isolates clustering together with *E. coli* from washed and unwashed spinach samples at retail at the similarity of at least 85.0%. This indicates that contamination that occurs on the farm can influence the safety of the final product at retail, regardless of processing steps (which often include washing in potable water) followed

through production. The importance of irrigation water as a contamination source of vegetables, in accordance with previous studies (Decol et al., 2017; du Plessis et al., 2015; Jongman & Korsten, 2016b), is further reiterated. Within the E. *coli* ERIC-PCR DNA fingerprint clusters generated for each supply chain, no specific pattern in phenotypic AMR profiles was established. To elucidate the AMR relatedness between these similar isolates throughout the respective supply chains, higher-resolved microbial typing through more sensitive methods such as whole-genome sequencing, should be included in future studies.

The results from this study provide valuable background information regarding the presence of multidrug-resistant environmental *E. coli* throughout spinach production from farm, during processing and up to retail. As AMR is a worldwide public health concern, surveillance of environmental bacteria as possible reservoirs in the water-plant-food interface becomes important. Furthermore, the necessity of using clean and safe irrigation water was highlighted with the need for standardized risk-based microbiological safety parameters for irrigation water of RTE fresh vegetables, as a link between *E. coli* from irrigation water and spinach at different points of the respective production systems were shown.

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CONFLICT OF INTEREST

No conflict of interest declared.

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