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Research Paper

Serotype Distribution, Antimicrobial Resistance, Virulence Genes, and Genetic Diversity of *Salmonella* spp. Isolated from small-scale Leafy Green Vegetable Supply Chains in South Africa

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ABSTRACT

Salmonella have been implicated in foodborne disease outbreaks globally and is a pressing concern in the South African small-scale sector due to inadequate hygiene standards and limited regulatory oversight, leading to a higher risk of foodborne diseases. By investigating irrigation water and leafy green vegetables produced by small-scale growers and sold through unregulated supply chains, this study was able to determine the presence, serotype distribution, virulence gene profiles, antibiotic resistance, and genetic diversity of *Salmonella* isolated from these sources. From 426 samples, 21 *Salmonella*-positive samples were identified, providing 53 *Salmonella* isolates. Of these, six different *Salmonella* serotypes and sequence types (STs) were identified, including *Salmonella* II 42:r: ST1208 (33.96%; n = 18), *Salmonella* Enteritidis: ST11 (22.64%; n = 12), *Salmonella* II 42:z29: ST4395 (16.98%; n = 9), *Salmonella* Havana: ST1524 (15.09%; n = 8), *Salmonella* Typhimurium: ST19 (9.43%; n = 5), and *Salmonella* IIIb 47:i:z: ST7890 (1.89%; n = 1). A total of 92.45% of the isolates were found to be multidrug-resistant, showing high rates of resistance to aztreonam (88.68%; n = 47), ceftazidime (86.79%; n = 46), nalidixic acid (77.36%; n = 41), cefotaxime (75.47%; n = 40), cefepime (71.70%; n = 38), and streptomycin (69.81%; n = 37). All isolates possessed the *aac(6')-Iaa* antimicrobial resistance gene, with a range of between 9 and 256 virulence genes. Eleven cluster patterns were observed from Enterobacterial Repetitive Intergenic Consensus sequence analyses, demonstrating high diversity among the *Salmonella* spp., with water and fresh produce isolates clustering, suggesting water as a potential contamination source. Plasmid replicon types were identified in 41.51% (n = 22) of the isolates, including Col(pHAD28) in *Salmonella* Havana (5.66%; n = 3), Col156 in *Salmonella* II 42:z29:- (1.89%; n = 1) and both IncFIB(S) and IncFII(S) in *Salmonella* Enteritidis (22.64; n = 12), *Salmonella* Typhimurium (9.43%; n = 5), and *Salmonella* Havana (1.89%; n = 1). This study highlights the presence of multidrug-resistant and multivirulent *Salmonella* spp. in the small-scale leafy green vegetable supply chains, underscoring the need for the development of a "fit-for-purpose" food safety management system within this system.

Salmonella species belonging to the *Enterobacteriaceae* family are Gram-negative, rod-shaped bacterial pathogens which cause salmonellosis in humans and animals, and are mainly associated with gastroenteritis (Scallan et al., 2015; Scallan et al., 2011). This zoonotic pathogen is one of the top four foodborne pathogens causing disease outbreaks globally (World Health Organization [WHO], 2020). In this context, almost one in ten people contract a *Salmonella* spp. infection annually, with 33 million lives lost globally due to this disease (WHO, 2020). In humans, infection is commonly characterized by fever, diarrhea, abdominal pain, nausea, and vomiting (WHO, 2008). Although salmonellosis in generally healthy humans subsides without antibiotic therapy, severe cases or patients with compromised immune systems can require antibiotic treatment (Centers for Disease Control and Prevention [CDC], 2009; Fardsanei et al., 2018). In 2020, an estimated 7.8 million people in South Africa were living with human immunodeficiency virus (HIV) and between 15.9% and 38.1% of them live in urban and rural informal settlements (Gibbs et al., 2020), putting them at higher risk for severe infection upon exposure. In animals,

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Salmonella spp. can be part of the native intestinal flora and *Salmonella* spp. can be carried asymptomatically (Ferrari et al., 2019). The pathogen can therefore easily be shed through feces, contaminate body surfaces, and, ultimately, the environment such as soil, water, and field-grown crops (Arthurson et al., 2011; CDC, 2022; Lee et al., 2019).

Notably, *Salmonella* spp. are often transferred through the consumption of contaminated drinking water (Levantesi et al., 2012), fresh produce irrigated with contaminated water (Steele & Odumeru, 2004), or cross-contamination through improper handling or poor hygiene practices (Deblais et al., 2018). Studies have previously linked *Salmonella* spp. to food, including fresh produce, namely tomato (Gurtler et al., 2018) and leafy green vegetables (Najwa et al., 2015; Nguyen et al., 2021) and other animal-based foods such as beef, pork, poultry, and seafood (Ferrari et al., 2019). Thus far, over 2,600 serotypes of *Salmonella* spp. have been described within the farm-to-fork continuum (Jajere, 2019). In addition, the increasing occurrence of resistance and multidrug resistance of *Salmonella* spp. has been reported in the water-plant-food nexus (Gomba et al., 2016a; Jongman et al., 2017; Nair et al., 2018).

Resistance tracking of *Salmonella* spp. and other foodborne pathogens has been established in the United States of America (USA) through the National Antimicrobial Resistance Monitoring System, by comparing strains isolated from food-producing animals and raw retail meat (CDC, 2020). This one health approach provides information to assess the nature and magnitude of resistance in bacteria moving through the food supply chain and causing illnesses in humans (McDermott et al., 2016). Since the introduction of whole genome sequencing (WGS), it has become possible to characterize an individual microbe quicker and at reasonably lower costs to assess crucial genotype information such as antimicrobial resistance (AMR) and virulence genotypes (McDermott et al., 2016; Uelze et al., 2020). Whole genome sequencing can additionally elucidate the serotype and sequence type that *Salmonella* spp. belong to, assisting in determining the origin (Ibrahim & Morin, 2018). A meta-analysis of animal-based foods from five continents found that the most frequent serotypes causing human infections were *Salmonella* Typhimurium and *Salmonella* Enteritidis (Ferrari et al., 2019). These were the most reported *Salmonella* serotypes causing leafy green vegetable-associated outbreaks in the USA between 1973 and 2012 (Herman et al., 2015).

In South Africa, people living in informal settlements have access to a limited variety of food, of which fresh produce is an important source of nutrition. The most popular of these are dark leafy green vegetables, collectively referred to as morogo (Jansen Van Rensburg et al., 2007) and spinach. Morogo and spinach are mainly field grown or cultivated in small-scale production systems (Department of Agriculture, Forestry and Fisheries [DAFF], 2013). During cultivation, small-scale morogo farmers utilize irrigation water that is often contaminated with *Enterobacteriaceae* (Ijabadeniyi et al., 2011; Ratshilingano et al., 2022). Despite the population understanding the detriments of contaminated food consumption, literature on the prevalence of *Salmonella* spp. within South African small-scale leafy green vegetable supply chains are limited. At harvest, distribution, or sale, morogo and spinach can become contaminated through inappropriate contact surface sanitation (e.g. picking crates and display tables), poor personal hygiene/handling practices by pickers, packers, and sellers, who have limited access to ablution facilities or potable water (Machado-Moreira et al., 2019). Furthermore, the use of contaminated rinse-water during packing or trading by street vendors in the informal sector (Gunel et al., 2015) acts as another contamination source.

Therefore, this study was undertaken to assess the prevalence of *Salmonella* spp. in local leafy green vegetable supply chains and to determine the serotypes, virulence genes, antibiotic resistance, and genetic diversity profiles of isolates obtained from small-scale production systems.

Materials and Methods

Sample collection

This study was conducted over a two-year period (May 2017 to January 2019), visiting eight small-scale farms (Table 1) in the Brits and Delmas regions in South Africa. Where possible, farms were visited twice during different growing seasons. Upon availability, all fresh produce types were collected from different farming blocks of a farm, as well as from at least one point-of-sale (retailer, hawker, or local market). Fresh produce and composite soil samples were collected systematically per farming block. Each composite soil sample consisted of five individual rhizozone (soil surrounding the plant root) samples collected adjacent to each individual fresh produce sampled. Water was collected from a water source (dam, stream, or river) as well as at the point of irrigation (overhead or flood). Water and soil samples were collected in sterile bottles, and fresh produce was placed in paper bags. A total of 426 samples, comprising 100 water samples (n = 36 reservoir and n = 64 irrigation water), 37 composite soil samples, and 289 leafy green samples (n = 65 rape, n = 106 chinensis, n = 94 spinach, n = 16 kale and n = 8 chomolia), were collected during primary production and later at the point-of-sale. All collected samples were transported in cooler boxes back to the University of Pretoria Food Safety laboratory for immediate processing.

Salmonella spp. isolation and identification

A minimum of 50 g of each of the fresh produce samples was weighed into a sterile polyethylene bag with 150 mL peptone buffered water (PBW) (Merck). Water samples (1 L) were filtered through a 0.45 µm nitrocellulose membrane and the membrane was placed into 9 mL PBW. Soil samples (5 g from each composite sample) were added to 225 mL PBW.

Each PBW-sample mixture was incubated at 37°C for 24 h (h). All samples were enriched by culturing in Rappaport Vassiliadis (RV) broth (Oxoid), where 100 µL of the PBW sample mixture was added to 10 mL of RV broth and incubated at 42°C for 24 h. For isolation of *Salmonella* spp., the RV-enriched samples were cultured in duplicate onto Brilliance™ *Salmonella* agar (Oxoid) and Xylose Lysine Deoxycolate agar (Merck) and incubated at 37°C for 24 h, according to the South African Bureau of Standards (SABS) ISO 6579 protocol (Du Plessis et al., 2021). Isolates with typical *Salmonella* spp. colony morphology were further identified using Matrix Assisted Laser Desorption Ionisation Time of Flight mass spectrometry (MALDI-TOF) (Bruker) in combination with the Bruker Biotyper software and database (Standing et al., 2013).

Antibiotic susceptibility testing

The Kirby-Bauer disk diffusion method (Clinical and Laboratory Standards Institute [CLSI], 2019; European Committee of Antibiotic Susceptibility Testing [EUCAST], 2019) was followed in combination with 9 mL of brain heart infusion broth (Merck) incubated for 18–24 h and subsequent plating on Mueller–Hinton agar (Merck) and incubated for 24 h at 37°C. A total of 21 antibiotics spanning twelve antibiotic classes were tested (Table 2). *Escherichia coli* ATCC 25922 and *Salmonella enterica* subsp. *enterica* serotype Typhimurium ATCC 14028 were used as quality control organisms. The breakpoints were measured and compared to the CLSI (2019) and EUCAST (2019) guidelines. The intermediate resistance values were regarded as susceptible according to Ta et al. (2014). Antibiotic resistance data were visualized in BioNumerics software version 7.2 (Applied Maths). Isolates with resistance to three or more antibiotic classes were defined as multidrug-resistant (MDR) *Salmonella* spp.

Table 1
Details of the sampled farms, sample sources, and collection dates for this study

Sampling area	Geographical region	Farm water supply	Sampled Crops	Sampled water supply	End point seller	Owner	No. of trips	Sampling date (s)
Farm A	Delmas	Borehole	S, R, C, Ch	HD & P	I & F	Married couple	3	22/05/2017, 25/04/2018, 28/11/2018
Farm B	Brits	Borehole	S, R, C	B & Fl	F	Female	1	29/07/2017
Farm C	Brits	River	S, R, C	HD & Fl	I	Male	3	29/09/2017, 02/10/2017, 06/07/2018
Farm D	Delmas	Borehole	S, K, C, Ch	Sp	I & F	Male	2	07/11/2017, 24/05/2018
Farm E	Brits	Borehole	S, R, C	Sp	I & F	Female	2	07/03/2018, 16/01/2019
Farm F	Brits	Borehole	S, R, C	Str & Fl	I	Female	1	17/05/2018
Farm G	Delmas	Borehole	S, R, C	Sp	I	Male	2	28/07/2018, 28/11/2018
Farm H	Brits	Borehole	S, R, C	Sp & Fl	I	Female	1	09/10/2018

S = spinach, R = rape, C = chinensis, K = kale, Ch = chomolia, HD = holding dam, P = pivot, Sp = sprinkler, Str = stream I = informal, F = formal.

Table 2
Antibiotics used for *Salmonella* antibiotic resistance testing in this study

Antibiotic class	Antibiotic	Concentration (μg)	Abbreviation
Aminoglycosides	Amikacin	30	AK30C
	Gentamycin	10	GM10C
	Tobramycin	10	TN10C
	streptomycin	10	S10C
Beta-lactam combination agent (BLCA)	amoxicillin-clavulanic acid	30/10	AUG30C
Carbepenems	Etrapenen	10	ETP10C
	Imipenem	10	IMI10C
	Meropenem	10	MEM10C
Cephems	Ceftazidime	30	CAZ30C
	Cefotaxime	30	CTX30C
	Cefepime	30	CPM30C
Fluoroquinolones	Nalidixic acid	30	NA30C
	Ciprofloxacin	5	CIP5C
Folate pathway	Trimethoprim-sufamethoxazole	25	TS25C
Fosfomycin	Fosfomycin/ trometamol	200	FOT200C
Macrolide	Azithromycin	15	ATH115C
Monobactam	Aztreonam	30	ATM30C
Penicillins	Ampicillin	10	AP10C
Phenicol	Chloramphenicol	30	C30C
Tetracyclines	Tetracycline	30	T30C
	Tigeclyclin	15	TGC15C

Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR)

DNA templates used for ERIC-PCR were extracted using PrepMan™Ultra Sample Preparation Reagent (Thermo Fisher Scientific) following the manufacturer's instructions. ERIC-PCR fingerprints were profiled using ERIC1R (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC2 (5'-AAG TAA GTG ACT GGG TGA GCG-3') (Mohapatra et al., 2007). A 25 μL PCR amplification reaction comprised of 1× DreamTaq Master-mix (Thermo Fisher Scientific), 0.4 μM of each primer and DNA template (100–600 ng). Thermocycling conditions were as follows: 94°C for 4 min, 30 cycles of 94°C for 30 s, 40°C for 60 s and 72°C for 8 min, with a final extension at 72°C for 8 min. The resulting amplicons were separated at 45V in a 2% agarose gel stained with CondaSafe for 8 h. Electrophoresed gels were visualized using the Gel Doc™ XR+ system (Bio-Rad Laboratories Inc.) and Image Lab software (Life Science Research, Bio-Rad Laboratories Inc.). The gel results were analyzed using BioNumerics software version 7.2. The analysis was conducted using unweighted pair group method with arithmetic means (UPGMA), where percentage similarities of the ERIC-PCR amplicons were calculated, and a dendrogram was created utilizing Pearson's correlation coefficient and linkage algorithms. An 80% homology cut-off was applied to determine different clusters.

Whole-genome sequencing, contig assembly, and genome annotation

All *Salmonella* isolates were sent for sequencing on an Illumina MiSeq platform (250-bp, paired-end reads, Illumina, Inc.) housed at the United States Food and Drug Administration's Centre for Food Safety and Applied Nutrition. Whole-genome sequence (WGS) contigs for each strain were *de novo* assembled using CLC Genomics Workbench 11.1. The whole-genome shotgun project has been deposited in the US National Library of Medicine's GenBank under the accession numbers outlined in Supplementary Table 1.

Serotype prediction and in silico multilocus sequence typing phylogenetic analysis

Serotype prediction was conducted using *in silico* SeqSero 1.0 (Zhang et al., 2015) and CRISPR-SeqSero (Thompson et al., 2018). FASTA files from raw WGS data files were uploaded to the online SeqSero tool version 1.0 which predicted the *Salmonella* serotypes of the requested isolate. Moreover, *in silico* multilocus sequence typing (MLST) was performed based on information available in the *Salmonella enterica* MLST database contained within Enterobase (https://enterobase.warwick.ac.uk/species/index/senterica). Seven *Salmonella enterica* loci (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) were used for this analysis and used to assign numbers to alleles and sequence types (STs) (Supplementary Table 2).

In silico detection of antimicrobial resistance genes, plasmid replicon types, Salmonella pathogenicity islands, and virulence genes.

FASTA files generated from the WGS data were used as templates in ResFinder 4.1 (https://cge.cbs.dtu.dk/services/ResFinder/) (Kagambèga et al., 2018) available at the Centre of Genomic Epidemiology (http://www.genomicepidemiology.org) webserver to identify antimicrobial resistance genes. Plasmid replicon types were determined using PlasmidFinder 2.1 (https://cge.cbs.dtu.dk/services/PlasmidFinder/) (Carattoli et al., 2014), *Salmonella* pathogenicity islands using SPIFinder 2.0 (https://cge.cbs.dtu.dk/services/SPIFinder/) (Roer et al., 2016), Virulence genes were determined using MyDbFinder 2.0 tool (https://cge.cbs.dtu.dk/services/MyDbFinder/) (Vilela et al., 2020) utilizing the virulence factor database (VFDB) full dataset (setB) containing all genes related to known and predicted VFs obtained from http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi.

Results

Salmonella spp. presence

A total of 21 out of 426 samples (4.93%) tested positive for *Salmonella* spp. These were detected from 17/100 water samples (17.00%), where 9/17 were detected in reservoir water and 8/17 in flood irrigation water. Four out of 289 leafy green vegetable samples (1.38%) were contaminated with *Salmonella* spp., where 1/4 were found on spinach at harvest, as well as 1/4 found on rape at point-of-sale and 2/4 were found on chinensis at point-of-sale. All 21 samples with *Salmonella* spp. were collected from two farms (Farm C and Farm F) in one growing region situated within Brits.

Salmonella spp. isolations

A total of 53 *Salmonella* isolates were obtained from the 21 positive samples. In 2017, a total of 27 isolates were obtained from Farm C, with 12/27 isolates collected from the six-positive reservoir water samples and 1/27 isolated from the 6-positive flood irrigation water samples (Table 3). No *Salmonella* spp. were detected from fresh produce or soil in 2017. In 2018, a total of 17 isolates were obtained from Farm C. Only 4/17 isolates were obtained from the positive reservoir water sample, 8/17 isolates were obtained from the 2-positive flooding water samples and 5/17 isolates were obtained from the 2-positive fresh produce retail samples (Table 3). In the same year, 9 *Salmonella* isolates were obtained from Farm F. Five of the 9 isolates were obtained from 2 positive reservoir water samples, 2 of the 9 isolates were obtained from the 1-positive field spinach sample, and 2/9 isolates were obtained from the positive rape point-of-sale sample (Table 3).

Antibiotic susceptibility testing

The antibiotic classes to which isolates were predominantly resistant, included monobactam (88.68%), cephems (77.99%), and fluoroquinolone (70.75%) (Fig. 1). A total of 49/53 *Salmonella* isolates (92.45%) were classified as MDR (Fig. 2). The most common resistance pattern was ATM-CAZ-NA-CTX-CPM-S-CIP-ETP-MEM-TN, where at least 62.26% of the isolates showed resistance to these 10 antibiotics (Fig. 2). The following tested antibiotics demonstrated high (>80%) resistance levels: aztreonam (88.68%) and ceftazidime (86.79%) (Fig. 3). The lowest (<30%) resistance was seen against, amoxicillin/clavulanic acid (20.75%), azithromycin (16.98%), and tetracycline (11.32%) (Fig. 3). Chloramphenicol was determined to be the most effective antibiotic agent, with 96.22% of the *Salmonella* isolates susceptible, and only 3.77% demonstrating resistance (Fig. 3). Also, 92.45% of the isolates were susceptible to fosfomycin and imipenem.

Serotype distribution

Of the 27 isolates obtained from 12 water samples on Farm C in 2017, serotypes identified included *Salmonella* II 42:r (n = 17), *Salmonella* II 42:z29 (n = 8), *Salmonella* Enteritidis (n = 1), and *Salmonella* IIIb 47:i:z (n = 1). Farm C serotypes identified in 2018 comprised of *Salmonella* Typhimurium (n = 5), *Salmonella* II 42:r (n = 1), and *Salmonella* Enteritidis (n = 11). Farm F 2018 serotypes identified included *Salmonella* Havana (n = 8) and *Salmonella* II 42:z29 (n = 1).

Farm C (2017 and 2018 samples) revealed *Salmonella* II 42:r only in reservoir water (n = 8) and flooding irrigation water samples (n = 10). Serotype IIIb 47:i:z was the only serotype isolated from flooding irrigation water in Farm C for 2017. In addition, all *Salmonella* Typhimurium isolates were found in point-of-sale chinensis (n = 3) and rape (n = 2) from Farm C only. *Salmonella* Havana was only isolated from Farm F (2018 samples), in reservoir water (n = 4), field spinach (n = 2), and point-of-sale rape samples (n = 2).

Sequence type distribution

In silico MLST analysis revealed six sequence types (Supplementary Table 2). All *Salmonella* II 42:r:- were assigned ST1208 (18/53; 33.96%), *Salmonella* Enteritidis were assigned ST11 (12/53; 22.64%), *Salmonella* II 42:z29:- were assigned ST4395 (9/53; 16.98%), *Salmonella* Havana were assigned ST1524 (8/53; 15.09%), *Salmonella* Typhimurium as ST19 (5/53; 9.43%), and *Salmonella* IIIb 47:i:z as ST7890 (1/53; 1.89%).

Cluster analysis

Of the 53 *Salmonella* isolates, based on the 80% threshold for homology, eleven clusters were observed, with six isolates remaining unclustered (Fig. 2). Two *Salmonella* Havana serotypes were grouped in cluster F: Farm F spinach at harvest and irrigation water (Fig. 3). In cluster G, these two *Salmonella* Havana are grouped alongside Farm C *Salmonella* Enteritidis isolated from flood irrigation water.

Detection of antimicrobial resistance genes and plasmid replicons

All 53 isolates harbored the *aac(6')-Iaa* resistance gene which is known to confer resistance to aminoglycosides (Salipante & Hall, 2003). Three *Salmonella* Havana isolates, CFSAN095700, CFSAN095701, and CFSAN095704, harbored the *qnrB19* resistance gene known to confer quinolone resistence, and the plasmid Col (pHAD28) in the isolates that are known to harbor the *qnbr19* gene. Plasmid replicon Col156 was detected in *Salmonella* II 42:z29, strain CFSAN095702, while 18 isolates of *Salmonella* Enteritidis (12/53), *Salmonella* Typhimurium (5/53), and *Salmonella* Havana (1/53) were identified as harboring plasmid replicons, IncFIB(S) and IncFII(S) (Supplementary Table 4).

Table 3
A summary of 53 *Salmonella* spp. isolated from 21 samples of water and fresh produce samples from small-scale informal supply chains

Description	Farm C				Farm F	
	Sampling year 2017		Sampling year 2018		Sampling year 2018	
	Samples	Isolates	Samples	Isolates	Samples	Isolates
Total	12	27	5	17	4	9
Reservoir water	6	12	1	4	2	5
Irrigation water	6	15	2	8	–	–
Fresh produce in field	–	–	–	–	1	2
Fresh produce at point-of-sale	–	–	2	5	2	2

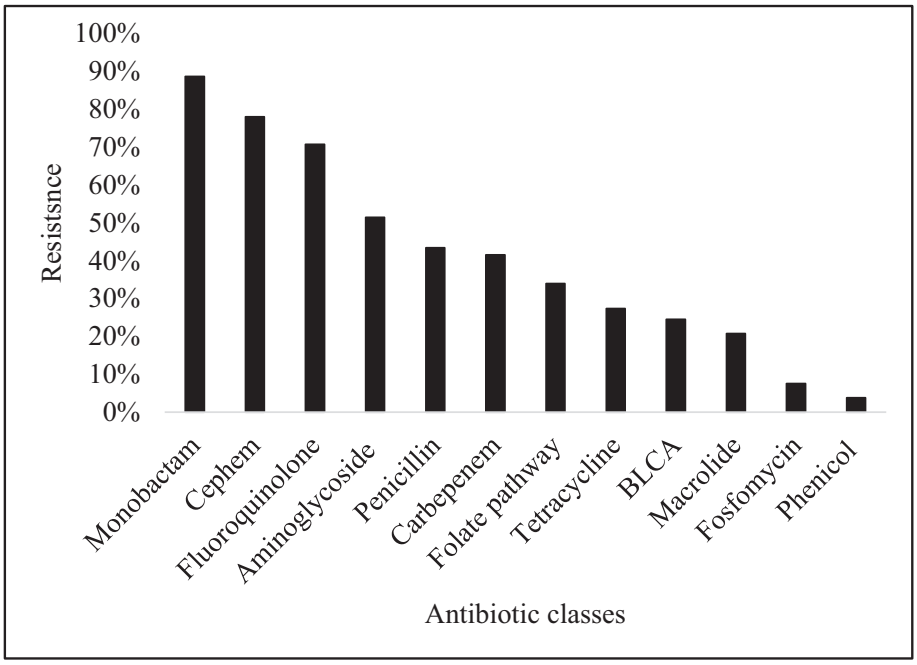

Figure 1. Antimicrobial resistance of *Salmonella* isolates to twelve antibiotic classes (in descending order).

Detection of Salmonella pathogenicity islands

All 53 isolates contained SPIs 1, 2, and 3. Additionally, 10 combination types could be deduced in this study (Supplementary Table 3) based on serotype specificity. From all 8 *Salmonella* Havana identified in this study, 5 isolates possessed C63PI; SPI1; SPI2; SPI3; SPI4; SPI5; SPI8; SPI9; UNNAMED, 1 isolate possessed C63PI; CS54_island; SPI1; SPI10; SPI13; SPI14; SPI2; SPI3; SPI5; SPI8; UNNAMED and 2 possessed C63PI; SPI1; SPI2; SPI3; SPI5; SPI8; SPI9; and UNNAMED (Supplementary Table 3). From the 12 *Salmonella* Enteritidis identified in this study, 10 isolates possessed C63PI; CS54_island; SPI1; SPI10; SPI13; SPI14; SPI2; SPI3; SPI4; SPI5; SPI9; UNNAMED, 1 contained C63PI; CS54_island; SPI1; SPI10; SPI13; SPI14; SPI2; SPI3; SPI4; SPI5; UNNAMED and another contained C63PI; SPI1; SPI10; SPI13; SPI14; SPI2; SPI3; SPI4; SPI5; SPI9; UNNAMED. All 5 *Salmonella* Typhimurium isolates identified in this study contained C63PI; CS54_island; SPI1; SPI13; SPI14; SPI2; SPI3; SPI4; SPI5; SPI9; UNNAMED. *Salmonella* IIIb 47:i:z contained C63PI; SPI1; SPI13; SPI2; SPI3; SPI5; SPI9. From the 18 identified *Salmonella* II 42:r:- and 9 *Salmonella* II 42:z29, 17 isolates and 8 isolates, respectively, possessed SESS-LEE; SPI1; SPI2; SPI3; SPI9, while 1 *Salmonella* II 42:r:- and 1 *Salmonella* II 42:z29 only possessed SPI1; SPI2; SPI3; SPI9.

Detection of virulence genes

A total of 256 virulence genes were identified in *Salmonella* Typhimurium (5/53) (Supplementary Table 5), 232 virulence genes in *Salmonella* Enteritidis (12/53) (Supplementary Table 6), 42 virulence genes in *Salmonella* II 42:z29 (8/53) (Supplementary Table 7), and in four isolates of *Salmonella* II 42:r (Supplementary Table 8), 34 virulence genes in twelve isolates of *Salmonella* II 42:r (12/53) (Supplementary Table 8), and nine virulence genes in *Salmonella* IIIb 47:i:z (1/53) (Supplementary Table 9). One *Salmonella* Havana (1/53) possessed 240 virulence genes, while seven had 231 virulence genes (Supplementary Table 10).

Discussion

The presence of *Salmonella* spp. in animals (Borges et al., 2013; Clemente et al., 2014; Deguenon et al., 2019; Mthembu et al., 2019; Ta et al., 2014; Zhao et al., 2017) and animal-based products (Beshiru et al., 2019; Chen et al., 2020; Ed-Dra et al., 2019; Hai et al., 2020; Han et al., 2020; Thung et al., 2018), as well as in fresh produce (Gomba et al., 2016a; Gu et al., 2018; Sant'Ana et al., 2011), have been widely reported. However, this is the first study to the authors' knowledge that considered the presence of *Salmonella* spp. on small-scale farming systems in South Africa, specifically cultivating dark leafy green vegetables and spinach. This study showed that water used for irrigation in the studied farming systems contained MDR *Salmonella* spp. Globally, the presence of *Salmonella* spp. in irrigation water is well documented and particularly important as a source of fresh produce contamination (Liu et al., 2018; Steele & Odumeru, 2004). This was confirmed in the present study when *Salmonella* Havana was found to be homologous in irrigation water (97.9% homogeneity) with that isolated from spinach at harvest, suggesting the possibility of irrigation water being a source of *Salmonella* contamination on fresh produce in farm C. As far as the authors were able to determine, this study is the first report of *Salmonella* Havana in irrigation water in South Africa and contaminating spinach in the field. This study also highlighted the predominance of *Salmonella* spp. contamination in source and irrigation water, dominated by *Salmonella* serotypes II 42:r, Enteritidis, II 42:Z29, and IIIb 47:i:z., which are of clinical importance (Breurec et al., 2019; Dione et al., 2011; Geimba et al., 2004).

Of the 53 isolates in this study, 33.96% were identified as *Salmonella* II 42:r species and were all from reservoir and irrigation water. Breurec et al. (2019) reported the isolation of *Salmonella* II 42:r from 2004 to 2010 in 2% of the human samples with 100% resistance to amoxicillin (Breurec et al., 2019) and Crump et al. (2021) reported *Salmonella* II 42:r:- ST1208 isolated from human, animal carcass, and meat (Crump et al., 2021). Although *Salmonella* II was not isolated from fresh produce in this study, *Salmonella* II have previously been detected in 2.81% of fresh produce in Mexico (Quiroz-Santiago et al., 2009). Thus, the risk of *Salmonella* II appearing on fresh produce

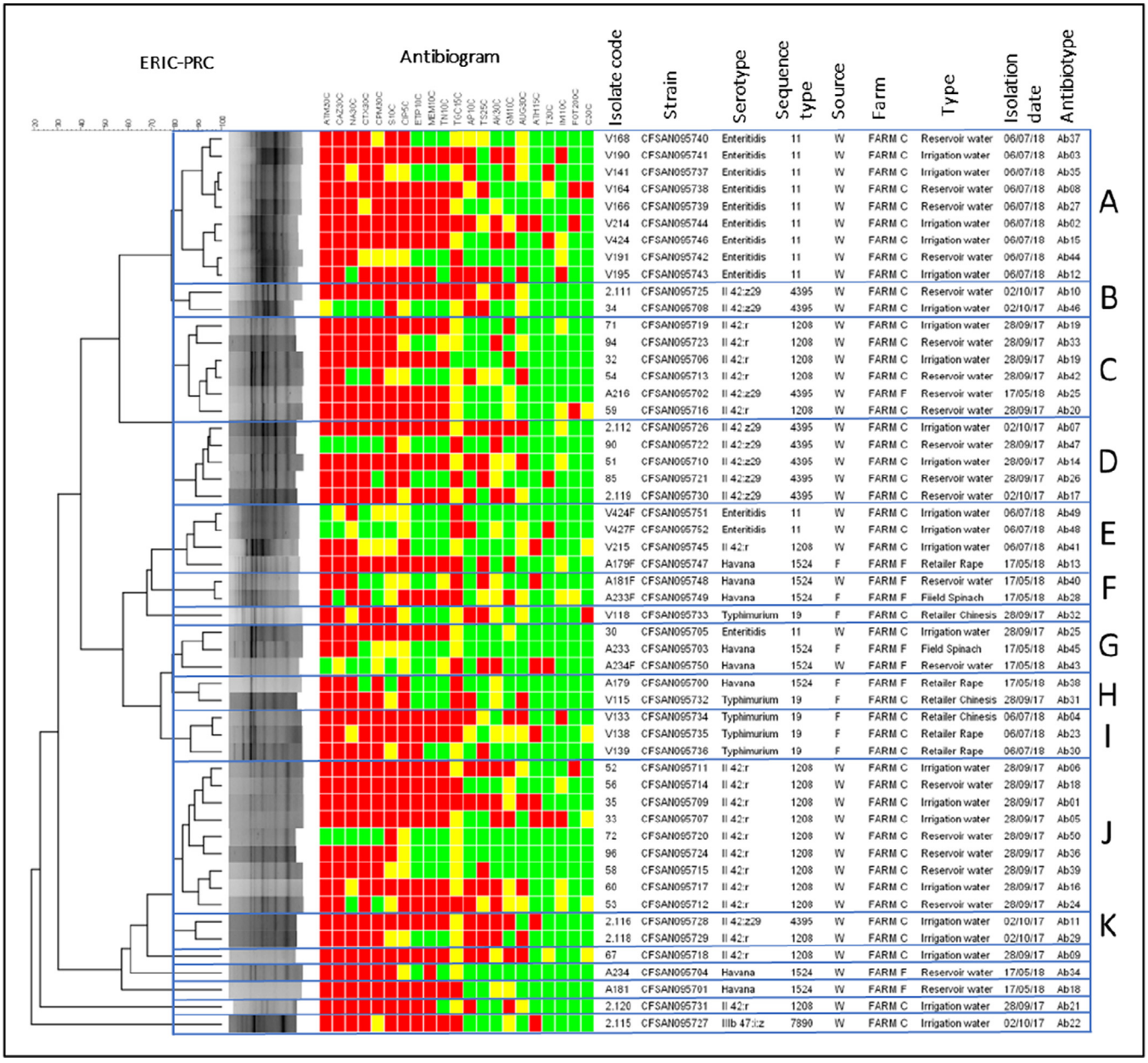

Figure 2. Dendogram of ERIC-PCR patterns of 53 *Salmonella* isolates from irrigation water and leafy green vegetables in South Africa showing antibiotic resistance phenotype (Antibiogram), isolate number, strain, serotype, sequence type, source: water (W) or fresh produce (F), farm, type, date of isolation, and antibiotic resistance pattern number (Ab). Red box indicates resistance, yellow intermediate, and green susceptibility to specified antibiotic.

is possible through contamination from irrigation water and may be detrimental to consumer health if the product is consumed minimally cooked, raw, dried, or without a cooking step, just as leafy green vegetables are consumed in households (Medoua & Oldewage-Theron, 2014). Although the prevalence of *Salmonella* spp. on morogo and spinach in this study was low (1.38%), it nevertheless highlights the health risk to consumers due to the presence of pathogenic *Salmonella* spp. on leafy green vegetables. *Salmonella* Havana has previously been implicated in severe and sometimes fatal outbreaks associated with alfalfa sprouts in California and Australia (Backer et al., 2000; Whitworth, 2018). In South Africa, between 2012 and 2014, the presence of *Salmonella* Havana was reported in poultry houses, abattoirs, and feed mills at 7.5% prevalence (Magwedere et al., 2015); however, it has not been associated with any reported outbreaks or fatalities.

In the current study, morogo at the point-of-sale was contaminated with *Salmonella* Typhimurium with 255 detected virulence genes, which can pose a potential risk to the consumer if consumed raw, such as in a salad. *Salmonella* Typhimurium has been one of the predominant serotypes identified in many countries and has been associated with large numbers of global foodborne disease outbreaks (Mohammed, 2017). For example, in Malaysia, *Salmonella* Typhimurium has been isolated from raw salad vegetables, including tomato (12%), carrot (20%), cabbage (16%), and lettuce (28%) (Nillian et al., 2011). Additionally, *Salmonella* Typhimurium has been associated with the fruit production environment in South Africa (Gomba et al., 2016a). As far as the authors could determine, foodborne outbreaks of *Salmonella* Typhimurium have not been reported in South Africa; however, two clinical-based outbreaks have been reported in a pediatric ward (Smith et al., 2014) and have been associated with meningitis (Keddy et al., 2015).

There are limited studies that investigate the AMR of *Salmonella* spp. isolated from fresh produce and irrigation water in South Africa

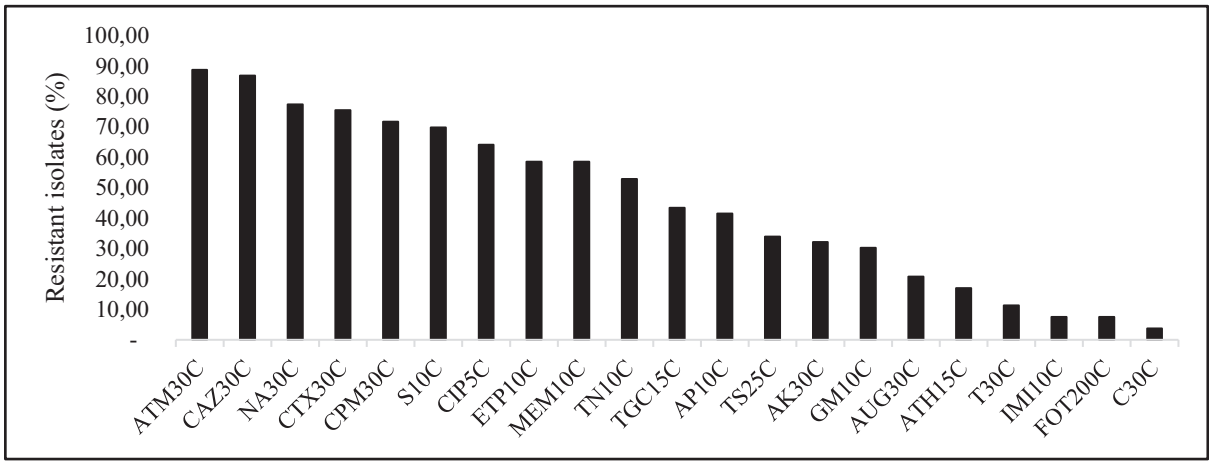

Figure 3. Percentage *Salmonella* spp. (n = 53) isolated from leafy greens supply chains that is resistant to 21 classes of antibiotics. Aztreonam (ATM30C), ceftazidime (CAZ30C), nalidixic acid (NA30C), cefotaxime (CTX30C), cefepime (CPM30C), streptomycin (S10C), ciprofloxacin (CIP5C). etrapenen (ETP10C), meropenem (MEM10C), tobramycin (TN10C), tigeclyclin (TGC15C), ampicillin (AP10C), trimethoprim-sufamethoxazole (TS25C), amikacin (AK30C), gentamycin (GM10C), amoxicillin-clavulanic acid (AUG30C), azithromycin (ATH15C), tetracycline (T30C), imipenem (IMI10C), fosfomycin/trometamol (FOT200C), and chloramphenicol (C30C).

and globally. The 92.45% presence rate of MDR in *Salmonella* spp. reported in this study was similar to that reported by Clemente et al. (2014), Fardsanei et al. (2018), and Nde & Logue (2008) in meat, poultry, and eggs. This shows the emergence of MDR *Salmonella* spp. in food production environments and hints to a serious health risk to consumers. We also demonstrated that similar serotypes do not necessarily show the same antibiogram patterns. Furthermore, we observed a rarely isolated serotype *Salmonella* IIIb 47:i:z which showed no MDR phenotype and showed resistance to only streptomycin. *Salmonella* IIIb encompasses Diarizonae strains regarded as pan–drug resistant bacteria (Bhatia et al., 2015). In this study, *Salmonella* IIIb 47:i:z was not found to be MDR, however, nine virulence genes were present within the isolate. Although *Salmonella* strains belonging to group IIIb (Diarozinae) have been identified as reptile-associated (Pedersen et al., 2009) and are regarded as a rare human-infecting *Salmonella* serotype (Bhatia et al., 2015), their potential to infect humans has been reported in India, where all affected patients were in close proximity to reptiles (Parihar et al., 2020).

Traditionally, the first line of antibiotics used to clinically treat *Salmonella* spp. infections in humans include streptomycin, ampicillin, trimethoprim/sulfamethoxazole, tetracycline, and chloramphenicol (Mthembu et al., 2019; Threlfall, 2002). High resistance to streptomycin was observed in 69.81% of the isolates in this study, which was contrary to Zishiri et al. (2016), which showed only 20% resistance from human clinical *Salmonella* isolates and Dione et al. (2011) which demonstrated 29.20% resistance. Moderate resistance to ampicillin (41.51%) and trimethoprim/sulfamethoxazole (33.96%) was observed, which corresponds to the 47.83% resistance illustrated by Thung et al. (2018) from *Salmonella* isolated from retail beef. Low resistance to tetracycline (11.32%) and chloramphenicol (3.77%) was observed; however, this result is in contrast to a study by Andoh et al. (2017) which showed 69% and 38% resistance to tetracycline and chloramphenicol, respectively. In a similar study done in South Africa in the fruit production industry, Gomba et al. (2016b) found that all *Salmonella* isolates were susceptible to tetracycline (100%) and moderately resistant to chloramphenicol (47%). Moreover, aztreonam, a clinical antibiotic used as an alternative to third-generation cephems such as ceftazidime (Brogden & Heel, 1986), revealed high resistant phenotypes to both antibiotics, 88.68% and 86.79%, respectively. This is contrary to resistant phenotypes of zero to both antibiotics as published by Ed-Dra et al. (2019) for *Salmonella* isolated from sausages. All isolates contained the *aac(6')-Iaa* resistance gene which is responsible for resistance to aminoglycoside antibiotics. The possession of this gene in all isolates is phenotypically reciprocated by the 46,23% resistance frequency in all isolates. Furthermore, *Salmonella* Havana isolates harboring the *qnrB19* resistance gene showed resistance to fluoroquinolone antibiotics, ciprofloxacin, and nalidixic acid.

The ERIC-PCR profiling of the *Salmonella* isolates belonging to the various serotypes showed high genetic diversity, revealing eleven clusters and six unclustered isolates. In this study, similar serotypes grouped in the same clusters (A, B, D, F, I, and J), which correlated to other studies (Fardsanei et al., 2017; Fardsanei et al., 2018) since *Salmonella* serotypes possess a degree of genetic homology. However, there were instances where different serotypes clustered together (C, E, G, H, and K). This was also observed in other studies where *Salmonella* Enteritidis, Pullorum, and Typhimurium in water and riverbed sediment clustered together (Ekwanzala et al., 2017), and for *Salmonella* Heidelberg and Typhimurium in the citrus production chain (Gomba et al., 2016b). Interestingly, only three *Salmonella* Typhimurium isolates clustered together while exhibiting different antibiograms, suggesting that although there is genetic homology among the isolates, the correlation to antibiotic resistance is unclear. A similar pattern can be extrapolated for cluster A, comprising only *Salmonella* Enteritidis exhibiting different antibiotypes. A similar non-specific serotype and antibiotype trend has been observed in Fardsanei et al., (2018). Despite the isolates being isolated from two different farms, the other *Salmonella* Typhimurium clustered with serotype *Salmonella* Havana in cluster H at 90.3% homogeneity, suggesting similar ancestor origins of the two. We also observed *Salmonella* II 42:r and *Salmonella* II 42:Z29 having similar genetic homology (clusters C and K). It is also noteworthy that all the isolates that did not show MDR phenotypes (CFSAN095720, CFSAN095722, CFSAN095751, CFSAN095752) cluster with isolates that exhibited MDR phenotypes in different clusters (D, E, and J), as observed by Chen et al. (2020). There is also evidence of 2017 and 2018 isolates clustering together, as indicated in clusters C, G, and H, suggesting that *Salmonella* from different seasons may have similar genetic homology, with the risk of replicating and transmission of virulence factors and MDR phenotypes in the environment, representing a concerning potential public health risk in South Africa. It is important for future studies to understand sources of contamination, levels of risk and development of mitigation strategies that will allow for the reduction of *Salmonella* spp. at farm level and point-of-sale. Farmer and trader education and aware-

ness are also important to ensure proper handling and preparation of produce to reduce the potential risk to the consumer.

Conclusion

This pioneering study on the presence of *S. enterica* serotypes in the morogo and spinach supply chain associated with small-scale production system highlight the clinical importance of their presence in the plant-food-nexus. The *Salmonella* isolates from fresh produce and water sources in this study harbor multiple AMR and virulence genes, with 92.45% being multidrug-resistant, highlighting the potential risk from the presence of pathogenic *Salmonella* spp. within the small-scale production system. The impact of these clinically important foodborne pathogens in South Africa should not be disregarded because they are not typically associated with foodborne outbreaks. As a large portion of the informal population within South Africa depends on dark leafy green vegetables such as morogo and spinach cultivated in small-scale production systems are also often immunocompromised and lack access to fresh nutritious food, this study provides an important framework for further work into determining the public health risks associated with the dissemination of MDR *Salmonella* spp. possessing aminoglycoside resistance genes and multiple virulence genes. Therefore, the development of a tailored food safety management system within small-scale primary production systems of leafy green vegetables is imperative for health and well-being of the people.

Ethical Statement

Ethical clearance was obtained for this research project, ethical clearance number: EC 180 327-182.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jfp.2023.100195.

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