Running title: Microbial quality and safety of spinach and cabbage

Research paper

Exploratory study into the microbiological quality of spinach and cabbage purchased from street vendors and retailers in Johannesburg, South Africa

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Abstract. Knowledge of the microbiological quality, prevalence of antibiotic resistance and virulence genes in bacterial isolates from leafy green vegetables supplied by formal (retailers) and informal (street vendors) suppliers in South Africa is limited. Since leafy vegetables have been implicated in food borne disease outbreaks world-wide, a total of 180 cabbage and spinach samples were collected from 3 major retailers and 9 street vendors in Johannesburg, SA. *Escherichia coli* and coliforms were enumerated using 3M Petrifilm count plates. The prevalence of *Listeria monocytogenes*, *Salmonella spp.* and *Shigella spp.* were determined using real-time PCR analysis. Identities of presumptive *E. coli* isolates from the fresh produce were confirmed using MALDI-TOF MS. Isolates were characterized using phenotypic (antibiotic resistance) and genotypic (phylogenetic, virulence gene) analysis. Hygiene indicator bacteria levels on spinach from formal and informal retailers exceeded the maximum level specified by the Department of Health guidelines for fresh fruit and vegetables. Street vendor spinach *E. coli* counts were higher ($p = < 0.0789$) than retailer spinach counts. *E. coli* was present in 2 cabbage samples only at 0.0035 CFU/g. *L. monocytogenes* and *Salmonella* spp. were detected in 7.2% and 5% of the 180 samples respectively using real-time PCR analysis, while Shigella was not detected. Of the 29 spinach *E. coli* isolates 37.9% were multi-drug resistant. Virulence genes *eae* and *stx1* were present in 14% and 3% of the spinach *E. coli* isolates respectively, while the *stx2* gene was not detected. Eighty-six percent (86%) of these isolates belonged to phylogroup A, 3% to group C, 7% to group E and 3% to clade 1. The results from the current exploratory study on the microbiological quality of spinach bought from selected retailers highlighted the need for continued surveillance on a larger scale, especially in the informal sector, in order to characterize the potential risk to the consumer.

Local fresh produce suppliers include informal street vendors, fresh produce markets, and a range of retail outlets or franchises. The Bureau of Market Research in South Africa reported that the informal retail sector, including street food vending, accounts for revenues of $2.4 billion (*45*). According to Consumers International it is estimated that street foods contribute up to 40% of the daily diet of urban consumers in developing countries (*8*). Handling practices during production and packaging of the fresh product are often questionable and the street vendors typically do not have any refrigeration facilities (*32*). A study in one of the local townships (Guguleto, Gauteng Province, SA) showed that spinach, cabbage and tomatoes are popular vegetables, especially for low-income families, as part of a healthy sustainable diet (*32*). Spinach (swiss chard) and cabbage are consumed cooked or are used raw in salads.

During 2008 the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) identified fresh produce as being the highest food safety risk in terms of microbial hazards associated with the product (*18*). The produce identified as the highest risk was leafy vegetables and leafy herbs followed by berries, greens onions, melons and sprouted seeds and tomatoes (*15*). From 2002 to 2014 there were six foodborne disease outbreaks linked to spinach consumption in the United States of America. This led to 300 illnesses, 114 hospitalizations and 6 deaths (http://wwwn.cdc.gov/foodborneoutbreaks/Default.aspx). Over the same period there were three outbreaks associated with cabbage consumption causing 72 people to become sick of which 8 were hospitalized and no deaths were recorded.

At a workshop hosted by the European Union FP7 Veg-i-Trade project in 2011 which aimed to capture opinions of stakeholders on food safety issues in the global supply chain, it was concluded that bacterial pathogens associated with fresh produce are an important food safety concern (*42*). Specific bacterial pathogens of most concern with regard to contaminated fresh produce include shiga toxin producing *Escherichia coli* (STEC)

serotypes, *Salmonella spp, Shigella spp., Listeria monocytogenes, Campylobacter spp.* and *Clostridium botulinum* (*23*). *E. coli* is an indicator of faecal contamination and have been implicated in nosocomial infections (*5*). Shiga toxin producing *E. coli* harbouring virulence-associated genes i.e. *stx1* (encoding shigatoxin1), *stx2* (encoding shigaroxin2) and *eae* (encoding intimin binding protein) have been linked to a number of foodborne disease outbreaks world-wide (*5*; *49*). The increased occurrence of potential human pathogenic bacteria as well as antibiotic resistance on leafy vegetables due to widespread use of antibiotics in agriculture, aquaculture and livestock industries, is a serious public health concern (*6*).

Clermont *et al.* (*11*) reported that *E. coli* has extensive genetic substructure and developed a phylo-typing method with improved specificity which showed that *E. coli* can be grouped into eight phylogroups (A, B1, B2, C, D, E and F) and the *E. coli* cryptic clade I. Strains from the designated groups belong to different ecological niches and differ in their disease causing ability and can facilitate tracking of *E. coli* contamination sources (*11*). For example, most commensal strains belong to *E. coli* group A and most virulent extra-intestinal strains belonged to group B2 and less often to group D. Meric *et al.* (*32*) observed that plant associated strains belonged predominantly to group B1, while group A and B2 were animal associated.

World-wide governing bodies, fresh produce industries and food processing companies adopted control measures, based on scientific data, to ensure the microbiological safety of food (*29*). In Canada, for example, targeted surveys of the microbiological quality of fresh produce at retail level contributed to a science based Food Safety Action Plan (FSAP) (*15*). However, to our knowledge there are no foodborne disease outbreak surveillance and reporting systems in South Africa. The current microbiological specification guidelines by the Department of Health (DoH) for ready-to-eat fresh fruit and vegetables was

published in 2000 and has since not been updated (16). This inevitably leaves a knowledge gap that requires updating.

There has been an increased focus on the potential contribution of food supply chains to the dissemination of antimicrobial resistance globally and the attribution of fresh produce to exposure of humans to multidrug resistant human pathogenic bacteria (*12*). Subsequently the aim of this exploratory study was to determine the microbiological quality (hygiene indicator bacteria) and prevalence of potential human pathogenic bacteria in leafy green vegetables sold by retailers (formal and informal) in Johannesburg, South Africa. The prevalence of antimicrobial resistance, virulence genes (*stx1*, *stx2* and *eae*) and phylogenetic groups in *E. coli* isolates were also determined.

Materials and methods

Suppliers and sampling. Samples were collected weekly from 9 street vendors (informal traders) and 3 retail chain outlets (formal traders) in Johannesburg, South Africa over a 2 month period from November 2014 to February 2015. Fifteen cabbage heads and 15 bunches of spinach were purchased from each retailer store (n= 45 cabbages and n= 45 bunches of spinach in total) over the period under investigation. Two branches were visited for retailers A and C and 3 branches for retailer B. The cabbages and spinach were selected randomly from display and purchased at the store as a fresh and ready to sell product. Due to limited supply of fresh produce at some of the street vendors at the time of sampling, cabbage (n=45) and spinach (n=45) were purchased from a total of eight different randomly selected street vendors (a-i) to determine the microbiological quality of the produce at the point of sale.

A sample comprised of one cabbage head or 1 spinach bunch. From each individual cabbage head, 3 leaves between layers 2 and 5 were obtained, mixed and a composite 25g

sample prepared. Leaves from spinach were obtained by aseptically cutting a section through the bunch with a sterelized knife, to include as many leaves as possible from each bunch, to make up individual 25g composite samples.

Microbiological analyses. From each produce sample, subsamples (25g) of leaf material were added to 225mL 3M buffered peptone water (3M) in a sterile polyethylene bag and macerated in a Stomacher 400 circulator (Seward Ltd., London, UK) at 135 rpm for five minutes. Ten-fold dilutions of each of the macerated fresh produce samples were prepared in buffered peptone water and total coliform and *E. coli* CFU/g determined by plating in duplicate onto *E. coli*/coliform count plates (3M Petrifilm, 3M, St. Paul, Minnesota, United States of America). Plates were incubated at 37°C for 18 h according to the manufacturer's instructions.

Selective enrichment for *Escherichia coli, Salmonella spp.* and *Listeria* spp. detection. For enrichment purposes the macerated spinach and cabbage samples in 3M buffered peptone water (3M Petrifilm, 3M, St. Paul, MN) ere incubated overnight at 37°C for detection of *E. coli* initially present at low numbers and *Salmonella* spp. Subsequently one ml of the enrichment broth was pelleted by centrifugation at 10 000 x g for 10 minutes for *Salmonella* spp. detection using Real-Time PCR analysis. A further 1mL of each respective subsample was added to Listeria broth (3M), supplemented with modified Listeria broth supplement (3M), and incubated overnight at 37°C. Subsequently 1 ml of each of the enriched samples were added to the corresponding sample pellet and centrifuged at 10 000 x g for 10 minutes. The nucleic acid of pelleted samples were extracted using the Quick-gDNA^{TM} Miniprep kit (Zymo Research, Irvine, CA) according to manufacturer's instructions.

Real-time PCR for the detection of *Salmonella* spp., *Shigella spp., Listeria monocytogenes.* Positive real-time PCR controls were prepared by spiking 25g sterile leaf

material in 225mL 3M buffered peptone water with cultures of the three target organisms: *L. monocytogenes* (ATCC 19115), *Shigella sonnei* (ATCC 25937) and *Salmonella enterica* subsp. enterica serovar Typhimurium (ATCC 14028) at a final concentration of 10^4 cells/ml. Cells were enumerated using a Petroff-Hausser counting chamber. Nucleic acid was extracted from the positive control samples as was described for the enriched spinach and cabbage samples.

Real-time PCR reactions were set up for each sample to determine the presence/absence of *L. monocytogenes*, *Salmonella* spp., *Shigella spp.*. The first real-time PCR reaction identified the Listeriolysin O (*hlyA*) gene in *L. monocytogenes* using the hlyA (F) 5"-TGCAAGTCCTAAGACGCCA-3'and hlyA (R) 5'-CACTGCATCTCCGTGGTATACTAA-3' primer set as well as the hlyA (P) 5'Cy 5-CGATTTCATCCGCGTGTTTCTTTTCG-BBQ-3'probe (*33*). An internal amplification control (IAC) was included using the IAC (F) 5'-ATGCCACGTAAGCGAAACA-3' and IAC (R) 5'-GCATAAACGAAGCAGTCGAGT-3' primer set as well as the IAC P 5'-*Cyan500* ACCTTACCGAAATCGGTACGGATACCGC-*BBQ*-3'probe (Roche diagnostics, Johannesburg , South Africa). The second PCR reaction identified the Invasion A (*invA*) and ipaH antigen (*shi/ipaH*) genes of *Salmonela* and *Shigella*, respectively. The *invA* gene was amplified using the invA (F) 5'- AATTATCGCCACGTTCGGGCAA-3' and invA (R) 5'-TCGCACCGTCAAAGGAACC- 3'primer set as well as the invA (P) 5'- Hex-TGGGTTTTGTTGTCTTCTCTATTGTCACCGTG-BBQ- 3'probe (*19*). The *shi/ipaH* gene was amplified using the Shi (F) 5'-CCTTTTCCGCGTTCCTTGA3 and Shi (R) 5'-CGGAATCCGGAGGTATTGC- 3' primer set as well as the shi (P) 5' FAM-CGCCTTTCCGATACCGTCTCTGCA-BBQ- 3' probe (*41*).

Each final PCR reaction mixture of 20µL contained 10µL LightCycler 480© Probes Master (Roche Diagnostics), 1µL of the relevant 20x primer/probe mix, 1µL IAC Lambda

DNA (Roche Diagnostics) and 8µL (50ng) DNA. Real-time PCR amplification was performed using a RotorGene Q instrument (Qiagen), equipped with RotorGene Q Series software (Qiagen) for data acquisition and analysis of results. The amplification was performed at 95°C for 10 min followed by 50 cycles of 10 sec at 95°C, 30 sec at 57°C and 1 sec at 60°C.

Isolation of *Escherichia coli*, *Salmonella* spp. and *Listeria* spp. using selective chromogenic media A loopfull of each of the enriched samples were streaked onto Eosin methylene blue differential agar (EMB) (Merck, Darmstadt, Germany) for isolation of typical *E. coli* colonies. Samples that tested positive for *Salmonella* spp. using real-time PCR analysis were streaked onto Brilliance TM Salmonella agar (SBM) for and Xylose Lysine Deoxycolate (XLD) agar (Oxoid Ltd, Basingstoke Hampshire, UK) for isolation of typical *Salmonella* colonies. Listeria positive samples were streaked onto Oxford-Listeria selective medium for isolation of typical Listeria colonies. Since *Shigella* was not detected in any of the fresh produce samples using real-time PCR analysis the isolation and identification of *Shigella* spp. were not further included in the study.

Confirmation of presumptive *Escherichia coli, Salmonella, Listeria* identities using Matrix Assisted Ionisation Time of Flight. Purified bacterial cultures isolated from the selective chromogenic media were transferred in duplicate directly to the Matrix Assisted Ionisation Time of Flight **(**MALDI-TOF) steel polished target plate (Bruker, Bremen, Germany), and overlaid with the α-cyano-4-hydroxycinnamic acid matrix (Bruker). The target plate was subsequently analysed using Bruker MicroFlex LT MALDI-TOF in conjunction with Bruker Biotyper Automation Software and library. The MALDI-TOF was calibrated prior to use with the bacterial standard supplied by Bruker. Duplicate score values (SV) were recorded; SV were used to determine the accuracy of identification. A SV of between 1.999 and 1.700 was used to identify the genus name of the organism, and a value of

above 2.0 was used to determine the genus and probable species of an organism. Above 2.3 is highly probable species identification.

Antibiotic resistance of *Escherichia coli* isolates. Twenty-nine *E. coli* isolates from spinach samples were subjected to antibiotic susceptibility testing using the Kirby-Bauer disk diffusion technique (*4*). Each *E. coli* isolate was cultured in 25 ml brain heart infusion broth,incubated for 24 h at 37°C and a 100 µl of each of the bacterial suspensions plated onto Mueller-Hinton agar plates (Merck). The antibiotic agents (22 in total) and concentrations used were summarized in Table 1 (Mast Diagnostics, Bootle, UK, supplied by Davies Diagnostics, Midrand, South Africa). Antibiotic resistance or susceptibility of *E. coli* isolates was scored according to criteria developed by the National Committee on Clinical Laboratory Standards (*14*).

Virulence genes in *Escherichia coli* isolates. The same *E. coli* isolates used for determining antibiotic resistance were tested for the presence of virulence genes including the *stx1* (shigatoxin 1) gene using stx1 (F) 5'-ACACTGGATGATCTCAGTGG-3' and stx1 (R) 5'-CTGAATCCCCCATTATG-3'; the *stx2* (shigatoxin 2) gene using stx2 (F) 5'-CCATGACAACGGACAGCAGTT-3' stx2 (R)-5' CCTGTCAACTGAGCACTTTG- 3'; the *eae* (intimin) gene using eae (F) 5'-CTGAACGGCGATTACGCGAA-3' and the house-keeping *mdh* gene (internal PCR reaction control) using mdh (F) 5'-GGTATGGATCGTTCCGACCT-3' and mdh (R) 5'-GGCAGAATGGTAACACCAGAGT-3' as described by Omar and Barnard (*35*). *E. coli* O157:H7 (ATCC 35150) was used as positive control for detecting the *eae*, *stx1* and *stx2* genes.

Assignment of *Escherichia coli* isolates into phylogenetic groups. Isolates were assigned to phylogroups by performing a quadruplex PCR based on the presence (+)/absence (-) of four genes i.e. 400 bp *arpA* gene (unknown function from an *E. coli* strain associated with neonatal meningitis), 288 bp *chuA* gene (a heme transport gene), 211 bp *yjaA* gene

Table 1 Summary of antibiotic agents and concentrations used in this study.

Antimicrobial class	Antimicrobial Agent	Abbreviation	Disk (µg/disk)
Aminoglycosides	Amikacin	AK30C	30
	Gentamycin	GM10C	10
	Kanamycin	K30C	30
	Streptomycin	S10C	10
	Neomycin	NE10	10
Tetracyclines	Tetracycline	T30C	30
β-Lactam/β-lactamase Inhibitor	Amoxicillin-clavunalate	AUG30C	20/10
Penicillins	Ampicillin	AP10C	10
Monobactams	Aztreonam	ATM30C	30
Cephalosporins	Cefepime	CPM30C	30
	Cefotaxime	CTX30C	30
	Cefoxitin	FOX30C	30
	Cefquinome	CEQ30C	30
	Ceftazidime	CAZ30C	30
	Ceftriaxone	CRO30C	30
Chloramphenicol	Chloramphenicol	C30C	30
Fluoroquinolones	Ciprofloxacin	CIP 5C	5
	Enrofloxacin	ENF5C	5
Quinolone	Nalidixic Acid	NA30C	30
Phenicol	Florfenicol	FFC30C	30
Tetracyclines	Tetracycline	T30C	30
Trimethoprim/ sulfonamides	Trimethoprim-sulfamethoxazole	TS25C	1.25/23.75

(sequence from the complete genome sequence of *E. coli* K-12 with function unknown) and 152 bp *TspE4.C2* (putative lipase esterase gene). The *ChuA* gene was amplified using chuA.1b (F) 5'ATGGTACCGGACGAACCAAC-3 and chuA.2 (R) 5′-TGCCGCCAGTACCAAAGACA-3′; the *yjaA* gene was amplified using yjaA.1b (F) 5′-CAAACGTGAAGTGTCAGGAG-3′ and yjaA.2b (R) 5′-AATGCGTTCCTCAACCTGTG-3′; the *TspE4.C2* DNA fragment was amplified using TspE4C2.1b (F) 5′-CACTATTCGTAAGGTCATCC-3′ and TspE4C2.2b (R) 5′-AGTTTATCGCTGCGGGTC GC-3′ (*9*). The 301 bp *arpA* gene was amplified using AceK.f (F) 5′-AACGCTATTCGCCAGCTTGC-3′ (*11*) and ArpA1.r (R) 5′-TCTCCCCATACCGTACGCTA (*10*). If an isolate was classified into group 'A or C' or group 'D or E' using the quadruplex primers described above a second PCR was performed to confirm that the isolate belongs to group E by amplification of a 301bp *arpA* gene using ArpAgpE.f 5′-GATTCCATCTTGTCAAAATATGCC-3′ and ArpAgpE.r 5′-GAAAAGAAAAAGAATTCCCAAGAG-3′ and group C the 219 bp *trpA* gene using trpAgpC.1 5′-AGTTTTATGCCCAGTGCGAG-3′ and trpAgpC.2 5′TCT GCG CCGGTC ACGCCC-3′ respectively (*27*).

Each PCR reaction contained 1x Bioline MyTaq™ reaction buffer (Bioline, Taunton, MA), 3 mmol of each primer, 1.5 U of Bioline MyTaq™ polymerase enzyme (Bioline) and approximately 80 ng of genomic DNA. The PCR was performed with a BioRad T100™ thermal cycler under the following conditions: denaturation for 4 minutes at 94°C; 35 cycles of 5 seconds at 94°C and 20 seconds at 59°C; and a final extension step of 5 minutes at 72°C. The PCR products were stained with GR Green DNA dye (Excellgenn, Inqaba Biotechnical Industries, Pretoria, SA) and 6x DNA loading dye (Thermo Scientific, Lituania, EU) and visualised on a 2% agarose gel using a BioRad Molecular Imager™ Gel Doc™ XR+.

Statistical Analyses. Data were analysed using SAS version 9.3 statistical software (*37*). The experiment was analysed as a factorial with the two factors the groups and the products. Differences between groups, products and the interaction were tested for in an analysis of variance. The Shapiro-Wilk test was performed on the standardized residuals to test for deviations from normality (*38*). Student's protected t-LSD (Least significant difference) were calculated at a 5% significance level to compare means of significant source effects (*39*).

Results

Microbiological analysis. The results obtained for the coliform and *E. coli* counts on spinach and cabbage from the retailers and street vendors were summarized in Table 2. The coliform counts on spinach from the retailers and street vendors were not significantly different. Similarly, the coliform counts on cabbage from the two supplier groups were not significantly different (Table 2). The coliform mean (4.78 log CFU/g) on spinach was significantly higher (p= 0.0005) than the coliform mean (3.65 log CFU/g) on cabbage (results not shown). All the spinach (n=90) and cabbage (n=90) samples from both formal retailers and informal street vendors were positive for coliform presence.

Comparison between the mean values of *E. coli* on spinach from street vendors (0.7896 CFU/g) were higher (p= < 0.0789) than the mean value on spinach from retailers (0.3684 CFU/g) (Table 2). Similar to the coliform counts, the *E. coli* mean (0.54 CFU/g) on spinach was significantly higher (p < 0.0001) than the *E. coli* mean on cabbage (0.0035 CFU/g). *E. coli* was present in 0-73% of the retailer spinach samples and 50-100% of vendor spinach samples.

Prevalence of *Escherichia coli, Salmonella, Shigella* spp. and *Listeria monocytogenes* in samples. The real-time PCR assays used for pathogen detection indicated

Table 2 Total coliform and *Escherichia coli* microbial loads (log CFU/g) present in spinach and cabbage purchased from various suppliers.

Product /sampling point (group)	**No. samples (% harbouring coliforms)**	**Total coliforms**		**No. samples (% harbouring *E. coli*)**	***E. coli***	
		Min-max	**Mean**		**Min-max**	**Mean**
Spinach	n=45			n=45		
Vendor a	2(100%)	3.85 - 5.89	4.9734^{A*}	2(100%)	0.25 - 0.48	0.7896^{A*}
Vendor b	2(100%)	4.95 - 5.89		2(100%)	1.02 - 1.52	
Vendor c	2(100%)	1.39 - 5.19		2(50.0%)	0.00 - 1.20	
Vendor d	13(100%)	4.49 - 6.00		13(53.8%)	0.00 - 1.74	
Vendor e	13(100%)	5.33 - 6.07		13(92.3%)	0.00 - 4.34	
Vendor f	13(100%)	TNTC*		13(100%)	0.34 - 4.31	
	n=45			n=45		
Retailer A	15(100%)	3.87 - 5.74	4.6446^{AB*}	15(73.3%)	0.00 - 1.34	0.3684^{B*}
Retailer B	15(100%)	2.64 - 5.60		15(26.6%)	0.00 - 1.85	
Retailer C	15(100%)	3.84 - 5.05		15(0.00%)	0.00 - 0.00	
Cabbage	n=45)			n=45		
Vendor a	6(100%)	5.48 - 5.73	4.0322^{BC*}	6(0.00%)	0.00 - 0.00	0.0037^{C*}
Vendor b	2(100%)	4.36 - 4.51		2(0.00%)	0.00 - 0.00	
Vendor c	2(100%)	5.11 - 5.26		2(0.00%)	0.00 - 0.00	

Vendor g	13(100%)	2.78 - 4.54		13(0.00%)	0.00 - 0.00	
Vendor h	9(100%)	4.59 - 5.00		9(0.00%)	0.00 - 0.146	
Vendor i	13(100%)	4.58 - 5.39		13(0.00%)	0.00 - 0.00	
	n=45			n=45		
Retailer A	15(100%)	0.95 - 5.99	3.3387[C*]	15(0.00%)	0.00 - 0.00	0.0034[C*]
Retailer B	15(100%)	1.97 - 4.53		15(6.66%)	0.00 - 0.146	
Retailer C	15(100%)	1.93 - 3.48		15(0.00%)	0.00 - 0.00	

* Values within columns followed by the same letters indicate that mean log CFU/g values based on the (group x product) statistic interactions are not significantly different (P<0.0).

that 7% and 5% of the 180 samples tested contained *L. monocytogenes* and *Salmonella* respectively. Shigella was not detected in any of the samples. Although presumptive *Salmonella* and *L. monocytogenes* were isolated using selective chromogenic media, subsequent analysis using MALDI-TOF showed that they were false positives.

Antibiotic resistance of *Escherichia coli* isolates. All the *E. coli* isolates (100%) were resistant to neomycin, 41% were resistant to trimethoprim-sulfamethoxazole, 31% to chloramphenicol, 20.7% to Streptomycin, 20.4% to Kanamycin, 13.8% to gentamycin and 13.8% to Ampicillin. Less than 10% of the strains were resistant to Aztreonam, Cefoxitin, Cefepime, Amoxicillin-clavulanate, Florfenicol, Ciprofloxacin and Enrofloxacin. None of the isolates showed resistance against ceftazidime, ceftriaxone, cefquinome, or cefotaxime which belongs to the Cephalosporin class of antibiotics.

The number of antimicrobials, most frequent resistance patterns, number and type of antibiotic classes to which *E. coli* isolates were resistant were summarised in Table 3. Eleven (37.9%) of the isolates can be regarded as multi-drug resistant as they were resistant to three up to eight classes of antibiotics (Table 3).

Virulence genes in *Escherichia coli* isolates. Virulence genes *eae* and *stx1* were detected in 14% and 3% the *E. coli* isolates respectively. None of the strains harbored both a shigatoxin gene (*stx1* and/or *stx 2*) and the intimin gene (*eae*) which would indicate the presence of a presumptive positive enterohemorrhagic *E. coli* (EHEC) strain. One of the strains carrying the *eae* gene grouped into *E. coli* phylogenetic group E. The *stx2* gene was not detected in any of the samples.

Phylogenetic group distribution. The same isolates that were used for determining antibiotic resistance were tested to determine which phylogenetic group each the isolates belong to. Phylogroup typing showed that 86% of these isolates belonged to phylogroup A (*arpA* +, *chuA* -, *yjaA* -, *TspE4.C2* -), 3% to phylogroup C (*arpA* +, *chuA* -, *yjaA* +, *TspE4.C2*

Table 3 Summary of the number of antimicrobials, most frequent patterns, number and type of antibiotic classes to which *Escherichia coli* isolates were resistant.

No of antimicrobials to which isolates were resistant	No of isolates (n=29)	No of isolates with specific pattern	Most frequent pattern	No of antibiotic classes to which isolates are resistant	Antibiotic class/ es
0	0				
1	11	11	NE 10C	1	Aminoglycosides
2	3	1	NEO 10C - TS 25C	2	Aminoglycosides Trimethiprim/sulphonamides
		1	NE 10C - GM 10C	1	Aminoglycosides
		1	NEO 10C - K 30C	1	Aminoglycosides
3	7	1	AUG 30C - ATM 30C- NEO 10C	2	β- lactamases; Monobactams
		2	NEO 10C - S 10C - TS 25C	2	Aminoglycosides; Trimethiprim/sulphonamides
		1	NEO10C; AP 10C - FFC 30C -	3	Aminoglycosides; Penicillins; Phenicols
		1	NE 10C; C 30C - GM 10C	2	Aminoglycosides Chloramphenicol
		1	NE 10C - FOX 30C – TS 25C	3	Aminoglycosides; Cephalosporins; Trimethiprim /sulphonamides
		1	NEO 10C - K 30C - C 30C	2	Aminoglycosides; Chloramphenicol
4	4	4	NEO 10C - K 30C - C 30C - TS 25C	3	Aminoglycosides; Chloramphenicol; Trimethiprim/ sulphonamides
5	2	1	NEO 10C - S 10C - C 30C - FOX	4	Aminoglycosides; Cephalosporins; Chloramphenicol;

			30C - CIP 5C		Fluoroquinolones
		1	GM 10C -TS 25C – NE 10C - CPM 10C - NE10C	3	Aminoglycosides; Cephalosporins; Trimethiprim/sulphonamides;
6	1	1	AP 10C – S 10C - GM 10C - TS 25C- ATM 30C - NEO 10C	4	Aminoglycosides; Monobactams; Penicillins; Trimethiprim sulphonamides
8	1	1	T 30C-Ap 10C ,C 30C- S 10C –TS 25C- ENF 5C – FFC 30C – NEO 10C	7	Aminoglycosides; Chloramphenicol ; Fluoroquinolone; Penicillins; Phenicols; Tetracyclines; Trimethiprim/ sulphonamides
9	1	1	T 30C-- NA 30C – AP 10C- C 30C - TS 25C – K 30C –ENF 5C- FFC 30C- NE 10C	7	Aminoglycosides; Fluoroquinolone; Penicillin; Phenicols, Quinolone; Tetracycline; Trimethiprim/sulphonamides

*Antibiotics abbreviations: GM10C:Gentamycin; Kanamycin:K30C; S10C:Streptomycin; NE10C:Neomycin; AUG30C:Amoxicillin-clavunalate; AP10C:Ampicillin; ATM30C:Aztreonam; FOX30C:Cefoxitin; CPM30C:Cefepime; C30C:Chloramphenicol; CIP5C:Ciprofloxacin; ENF5C:Enrofloxacin; NA30C: Nalidixic Acid; FFC30C:Florfenicol; T30C:Tetracycline; TS25C:Trimethoprim-sulfamethoxazole.

and phylogroup C specific *trpAgpC.1* +), 7% to phylogroup E (*arpA* +, *chuA* +, *yjaA* -, *TspE4.C2* - and *ArpAgpE* +) and 3% to clade 1 (*arpA* -, *chuA* -, *yjaA* +, *TspE4.C2* -).

Discussion

Globally several studies have focused on determining the microbiological quality of fresh produce sold at supermarkets, open air markets, green grocers and street vendors from selected areas or towns in a specific country (*26; 36; 49*). However, in South Africa microbiological surveillance data for fresh produce, especially from the informal sector is limited. This exploratory study provided usefull baseline information regarding the microbiological quality of fresh leafy vegetables sold at selected retailers and street vendors in Johannesburg South Africa. The guidelines for microbial *E. coli* count limits for fresh vegetables differ for each country. Current South African Department of Health (DoH) microbiological guidelines for fresh fruit and vegetables to be eaten raw require coliform counts of less than 200 (2.3 log CFU) per gram and no *E. coli* or *Salmonella* in 25g of sample (*16*). In this study all the spinach samples from retailers and street vendors tested positive for coliforms. Except for 1 sample from a street vendor, the coliform counts exceeded the maximum limit specified by the guidelines (*16*). In contrast to these results, a survey of fresh produce obtained from retail stores on the Eastern shore of Maryland in the United States of America (USA) showed that only 17.4% to 26.1% of spinach samples were positive for coliforms (*26*). The coliform counts on spinach in this study were similar to the results obtained for baby spinach (2.0 log CFU/g to 5 log CFU/g) from a farm and processing facility supplying retailers in the formal sector in South Africa (25). In contrast the coliform counts for spinach obtained from supermarkets in Istanbul, Turkey ranged between 2.95 log CFU/g to 3.45 log CFU/g which was approximately 1.5 log CFU/g to 2.5 log CFU/g lower that the maximum coliform counts measured for spinach from formal and informal suppliers

in this study (*7*). In a recent study by Xu *et al.* (*47*) a total coliform mean value of 6 log CFU/g was measured for leafy green vegetables (lettuce, spinach, Swiss chard and salad greens) which is comparable to the to the spinach mean values of 4.6446 log CFU/g (retailer samples) and 4.9734 log CFU/g (street vendor samples) measured in the current investigation. The mean values of total coliforms on spinach were reported to be similar to a variety of fresh produce including basil, cilantro, lettuce, scallion, and parsley in a study by Korir *et al.* (*26*). However in this study statistical analysis showed a significant difference between the mean coliform as well as the *E. coli* levels on spinach when compared to cabbage.

In the study by Jongman and Korsten (*25*) *E. coli* colonies were enumerated from of 18, 20, and 27% for baby spinach, lettuce and cabbage, respectively. In our study *E. coli* colonies were enumerated from 0-73.3% retailer spinach samples and 50-100% street vendor spinach samples. *E. coli* colonies were enumerated from only 3.33% and 6.66% of cabbage samples from one retailer and one street vendor respectively which is much lower than the 27% reported in the earlier study on a commercial farm in SA. Korir *et al*. (*26*) reported that the *E. coli* counts on spinach from retailers ranged between <1.0 log CFU/g to 1.78 log CFU/g which is similar to results obtained for the retailer and most of the street vendor spinach samples analysed in this study. However, at two of the street vendors *E. coli* counts of up to 4.34 log CFU/g were measured which is regarded as an indicator of faecal contamination during production and/or processing. Informal street vendors often do not have access to cooling facilities, ablution facilities and a clean safe water source for drinking and hand washing purposes. Al-Kharousi *et al*. (*1*) reported a mean *E. coli* count of 3.1 log CFU/g on cabbage samples, while in our study *E. coli* levels < 10 CFU/g were measured for cabbage samples in this study.

The presence of *E. coli* on spinach from 2 of the 3 retailers as well as most of the street vendors showed that the microbiological quality of the vegetables were unacceptable according to DoH guidelines, since the guidelines stipulate that *E. coli* should be absent from fresh produce likely to be eaten raw. If measured against other guidelines as specified in the United Kingdom (20-100 *E. coli* CFU/g), Australia (3-100 CFU/g) and Canada (100 MPN *E. coli*/g), 86% of the spinach samples would have been compliant (*20*; *21*; *34*). Furthermore, a global trend is to exclude coliform specifications as in Hong Kong (*2*) and Australia (*34*). The Canadian guidelines states that high levels of coliforms should be expected in any raw produce (*21*).

In this study, 86% of the *E. coli* isolates were classified into phylogenetic group A according to the revised Clermont method (*11*). In contrast, Wood *et al.* (46) reported that 79% of the isolates, originating from locally grown lettuce in Vancouver, were classified into phylo-group B2, and 12% and 9% belonged to phylo-groups D and A, respectively. The author also proposed that only phylo-groups A, B2 and D are truly suggestive of faecal contamination (*46*). In humans, *E. coli* strains of phylo-group A and B2 are predominant, though large variation of prevalence is found among different populations (*40*). For example, the prevalence of group A in Michigan, USA, is only 20.5% ,while the prevalence of the same group is 63.7% in French Guiana (*3*; *40*). Meric *et al.* (*31*) reported that *E. coli* strains associated with plants were more likely to belong to phylogroup B1. The prevalence of *E. coli* strains found on produce could vary according to produce type, geographical location, and possibly human population, although further research would be required to prove the hypothesis.

Identification of potential STEC positive samples by initial screening for STEC markers (*stx1*, *stx2* and *eae* genes) is usefull for food safety surveys, however isolates should be culturally recovered and strain/s confirmed to be *E. coli* O157: H7 or non- *E. coli*

O157:H7 (*28*). Li *et al.* (*28*) reported that 5.3 % of spinach samples harbored the *stx2* virulence gene, while it was not detected in any of the spinach samples analysed during our study. In the study by Jongman and Korsten (*25*) none of the STEC virulence markers were detected in any of the spinach samples.

The spinach *E. coli* isolates were susceptable to 2nd generation (cefoxitin) and 3rd generation cephalosporin antibiotics (ceftazidime, ceftriaxone) in our study which is in agreement with results for spinach samples from a large-scale commercial farm with an on-farm packing house in Gauteng Province in South Africa (*24*). Walia et al. (*44*) reported that 95.3 % gram negative bacteria from ready-to-eat baby spinach were resistant to two or more antibiotics, while 37.9% of *E. coli* isolates from our study were multidrug resistant to between three and seven different antibiotic classes (i.e. between three and nine antibiotics). Antibiotic resistance genes are also regarded as emerging environmental contaminants, since the genes can be transferred easily to commensal bacteria which typically colonises the human gut (*13*; *30*). The use of polluted water sources for irrigating produce may pose a risk of transferring human pathogenic bacteria onto crops, especially those which undergo minimal postharvest processing (*42*). A number of studies in South Africa specifically showed that *E. coli* in irrigation water can be transferred onto produce in the field (*17*, *22*, *25*).

The results from the current exploratory study on the microbiological quality of spinach bought from selected retailers highlighted the need for continued surveillance studies on a larger scale, especially in the informal sector, in order to characterize the potential risk to the consumer. Knowledge of the microbiological quality, potential contamination sources i.e. irrigation water, soil, manure and post harvest processing will contribute to developing South African crop-specific supply-chain management systems and guidelines aligned with international guidelines.

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