

Prevalence and serovar diversity of *Salmonella* spp. in primary horticultural fruit production environments

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Abstract

Increases in foodborne disease outbreaks associated with fresh produce have necessitated the need to identify potential sources of microbial contamination in produce and agricultural environments. The present study evaluated *Salmonella* prevalence and serovar diversity in fruit (225), water (140) and surface (126) samples, from three commercial farms and associated packhouses, located in different farming regions in South Africa. Fruit and water samples were collected from both orchards and packhouses, while surface samples were collected from conveyer belts and hands of packhouse employees. *Salmonella* was detected in 26 of the 491 (5.3%) samples. Environmental samples (water and surfaces) recorded a slightly higher proportion (3.1%; 15/491) of positive samples compared to fruit samples (2.2%; 11/491). *Salmonella* was not detected on employee hands and river water samples. A total of 263 *Salmonella* isolates were obtained from the 26 positive samples by standard culture methods, preliminarily identified through matrix-assisted laser desorption ionisation-time of flight mass spectroscopy (MALDI-TOF MS) and API 20E, and confirmed by *invA* gene. Of the 39 representative isolates serotyped the serovars Muenchen (33.3%), Typhimurium (30.8%), Heidelberg (20.5%), Bsilla (7.7%), *Salmonella* subspecies IIb: 17: r: z (5.1%) and one untypable strain were identified. Most samples had multiple serovars with orchard water from one site recording the highest serovar diversity (4 serovars). Our findings

show the potential of agricultural fruit production environments to act as reservoirs of clinically important *Salmonella* serovars.

Key Words: *Salmonella*, prevalence, serovars, primary production, packhouse sanitation

1. Introduction

The consumption of fresh produce has significantly increased along with the promotion of a healthy diet (Sivapalasingam, Friedman, Cohen & Tauxe, 2004). This has led to increased risks associated with foodborne disease outbreaks of which *Salmonella* has been identified as the most important pathogen in the European Union and United States (Callejon et al., 2015). Although foodborne disease outbreaks are commonly associated with foods of animal origin, fresh produce has been linked to 46% of annual foodborne illnesses, 38% hospitalisations and 23% deaths in the United States alone for the period between 1998-2008 (Painter et al., 2013). However, data on microbiological food safety of fresh produce particularly fruit in developing countries is lacking.

Fruits are generally considered a low risk food commodity because they usually are a tree crop and are therefore not directly exposed to soil and irrigation water, the principal sources of contamination at the preharvest phase (Beuchat & Ryu, 1997). However, orchard fruit can come into direct contact with insufficiently treated water used to reconstitute pesticides and other foliar agro-chemicals (Lopez-Velasco, Tomas-Callejas, Diribsa, Wei, & Suslow, 2013; Ng, Fleet & Heard, 2005). Moreover, contaminated soil from stagnant shallow pools following damaged irrigation line leaks and rain events can be deposited on low hanging fruit through splashes (Micallef et al., 2012). Orchards attract wildlife including birds and large animals. The presence of animals in agricultural production environments have been cited as a significant source of contamination on crops in the preharvest phase (Nielsen et al., 2004; Langholz & Jay-Russel, 2013).

A number of salmonellosis outbreaks linked to contaminated fruit grown in orchards have been reported with sources of contamination routinely traced back to production (field) and processing (packhouse) environments (Strawn et al., 2013). For example, a *Salmonella* Enteritidis phage type (PT) 30 implicated in raw almond *Salmonella* infection in Canada during 2000 and 2001 was detected in environmental surfaces of associated farmers' orchards

and processing equipment (Isaacs et al., 2005). In a separate study, the same strain was repeatedly isolated from orchard floors of implicated farms in a large agricultural area suggesting a widespread contamination source in these environments (Uesugi, Danyluk, Mandrell, & Harris, 2007). A multistate outbreak of infection with *Salmonella* enterica serotype Newport linked to consumption of mangoes was traced back to mangoes imported from a single packing house in Brazil (Sivapalasingam et al., 2003). The presence of *Salmonella* in horticultural environments may present a more serious public health concern than previously thought given that the pathogen is ubiquitous in nature and has been demonstrated to persist and sometimes grow in non-host environments such as water, soil, plants and packing environments (Maurer et al., 2015; Waldner, MacKenzie, Köster & White, 2012).

The genus *Salmonella* comprises two distinct species, *S. bongori* and *S. enterica* with over 2,500 *Salmonella* known serovars (Grimont & Weill, 2007; Su et al., 2007). Although all serovars are potentially pathogenic, only a limited number belonging to *Salmonella enterica* subsp. *enterica* are of public health importance with disease manifestation depending on serovar involved (Ktsoyan et al., 2013). Studies have demonstrated a link between specific *Salmonella* serovars and reservoirs, serovars and food commodities (Jackson, Griffin, Cole, Walsh, & Chai, 2013) and unique serovar prevalence in different geographical regions (Hendriksen, 2003; Maurer et al., 2015). Therefore, *Salmonella* serotyping may provide information on potential links between contamination routes and risks from the consumption of contaminated produce.

Given that fresh fruit can be contaminated by *Salmonella* at any point along the production chain and that they are generally consumed raw without prior treatment to kill pathogens, the need for more research to establish occurrence, reservoirs and serovar diversity in farm operation environments becomes very important. This information is critical in designing effective control strategies to reduce fruit contamination and in assessing the potential impact of recovered *Salmonella* strains on human health. This study was undertaken to determine the prevalence, potential contamination sources and serovar diversity of *Salmonella* on fruit, in agricultural contact water and environmental samples from the field (production) through packhouse processing. Additionally, the prevalence of the invasive gene (*invA* gene) in recovered isolates was also determined.

2. Materials and methods

2.1. Site description

Samples were collected during 2011 from three commercial farms and associated packhouses hereafter designated as sites A, B and C. The types of fruit produced on these farms as well as the exact locations are not given due to confidentiality reasons. Moreover, it was not the purpose of this study to investigate a specific fruit type but rather a horticultural production system. Each farm had an on-site packhouse with similar packing procedures except for the washing step. Packhouse A used a dry system where unwashed fruit was conveyed directly into the packhouse and then received a chlorine spray (150-200 ppm total chlorine) once inside. Packhouse B used a wet system whereby unwashed fruit was drenched with high pressure water with no sanitising agent just before entering the packhouse. Packhouses C used a wet system whereby unwashed fruit was received into a chlorine dump tank (150-200 ppm total chlorine) outside the packhouse. All four farms and packhouses were GlobalGAP certified. Sampling was repeated at site A and B (early and late season) whereas for site C it was done once during the late harvesting season.

2.2. Sample collection

On each farm two orchards were randomly selected and preharvest low hanging fruit were collected from five randomly selected trees in each orchard. At the packhouse, fruit samples were collected from harvesting crates before wash and at various points along the packline including after washing, fungicide treatment, wax application and in the final packed box. Using gloved hands, and changing gloves for each sample, samples were collected in triplicate at each sampling point with each sample consisting of three pooled fruit placed in sterile paper bags.

Environmental samples included water and palms of sorters and packers hands and packline surfaces. Water samples were collected from both pre- and postharvest stages at each farm and the associated packhouses and included river, dam or storage tank, orchard irrigation pipe, wash, fungicide warm bath and tap water. Water samples were collected in sterile 1 L plastic screw-cap bottles in five replicates per sampling point. Surface samples were collected with sterile swabs (Medical Wire and Equipment, Johannesburg) from hands of packhouse personnel and approximately 25 cm² of conveyer belt that came into contact with fruit. Surfaces were thoroughly swabbed with at least ten passes vertically and horizontally. In total 225 fruit samples, 140 water samples and 126 surface samples (packline and hands) were

collected (Table 1). All samples were collected aseptically, were clearly labelled and placed in separate sterile cooler boxes with ice packs and transported to the Plant Pathology Laboratories, University of Pretoria. Samples were kept chilled at 4 °C for no longer than 48 h prior to analysis.

2.3. *Salmonella* detection and isolation

All microbiological media were purchased from Merck, South Africa unless otherwise stated. All samples ($n=491$) were analysed for *Salmonella* following the United States Food and Drug Administration (U. S. FDA) Bacteriological Analytical Manual (BAM) protocol for fresh produce (Andrews and Hammack, 2007) with minor modifications to enable the enumeration of total microbial load (aerobic bacteria, fungi and yeasts) from the samples and the results will be reported elsewhere.

Fruit samples (three fruit per sample) were transferred into a 2 L sterile glass beaker containing 1 L sterile 0.1% buffered peptone water supplemented with 0.02% (v/v) Tween 80 (Associated Chemical Enterprises, Johannesburg). Microbial epiphytes on fruit surfaces were dislodged by sonication in a digital heated ultra-sonic cleaner (Eumax, UD200SH-6L, Labotec, Johannesburg) for 5 min at 200W and 50Hz. The microflora washings were concentrated by filtration through a 0.45µm pore-size nitro-cellulose membrane (Sartorius, Goettingen, Germany). Water samples (1 L) were also filtered as described for fruit microflora washings. All materials and equipment for sample processing (filtration cups, beakers and tweezers, etc.) were sterilised by immersion into boiling water for a minimum of two minutes between samples in order to prevent cross-contamination.

Following filtration, the membranes were aseptically transferred into 9 ml tryptone soy broth (TSB) and incubated overnight with agitation at 150 rpm for 24 h at 37 °C. Subsequently, 0.1 ml of the pre-enriched broth was inoculated into 10 ml Rappaport for selective enrichment of *Salmonella* and incubated with shaking (150 rpm) at 42 °C for 24 h. A loopful of each selective enrichment culture showing growth was streaked onto Xylose Lysine Deoxycholate (XLD) agar plates and incubated at 37 °C for 24 h. Gorski et al., (2011) reported that XLD and Hektoen enteric (HE) agars recommended by USFDA BAM for *Salmonella* isolation give identical results and we therefore opted for the former.

All isolates with typical colony morphology (pink colonies with black centres) for *Salmonella* on XLD agar plates were picked and streaked for purification. Although most researchers isolate a maximum of 3-10 presumptive colonies for further analysis and characterisation we isolated a maximum of 20-25 colonies from each positive sample to enhance the chances of capturing a wide diversity of *Salmonella* strains (Strawn et al., 2014). Pure single colonies were then streaked on Standard 1 (STD 1) nutrient agar and maintained as working cultures at 4 °C. A glycerol stock (35%) of each pure isolate was also prepared from overnight TSB enrichment cultures and stored at -70 °C for future work. At the same time, 3 ml of the overnight enrichment pure culture was centrifuged (9 000 rpm for 5 min at 4 °C) to obtain a pellet of viable cells and stored at -20 °C until required for gDNA extraction as described below.

2.4. Identification of presumptive Salmonella isolates using MALDI TOF-MS

A single pure colony of each isolate on XLD agar was streaked on STD 1 nutrient agar and incubated at 37 °C for 24 h. Cultures were prepared and identified from the 24 h colonies without extraction according to manufacturer instructions (Bruker Daltonik, Bremen, Germany). Mass spectral fingerprint of unknown bacteria isolates were automatically matched against the reference library by means of an integrated pattern-recognition algorithm software. The degree of spectral pattern matching is expressed as a logarithmic identification score and interpreted according to the manufacturer's instructions. MALDI-TOF identification results were recorded as score values (SV) and used to determine the identity of the organism as proposed by the manufacturer. A score <1.700 indicates no identification, 1.700-1.999 indicate genus, ≥ 2.000 denotes genus and probable species identification and above 2.3 is highly probable species identification.

2.5. Analytical Profile Index (API 20E) test

All isolates that were identified as *Salmonella* spp. using MALDI-TOF MS were further tested by the API 20E kit according to manufacturer's protocol (BioMérieux, Marcy-l' Etoile, France) for biochemical confirmation of *Salmonella*. Results of the phenotypic profiles were interpreted using the API web software.

2.6. DNA extraction and PCR confirmation of Salmonella isolates by invA gene detection

Frozen pellets of pure isolates putatively identified as *Salmonella* spp. using both MALDI-TOF MS and API 20E were thawed and the DNA was extracted using the Quick-DNA

MiniPrep kit (Zymo Research, Irvine, California, USA) according to the manufacturer's instructions. Eluted DNA was quantified using the Qubit 2.0 Fluorometer (Life Science Technology, Johannesburg). Polymerase chain reaction (PCR) was conducted for all putative *Salmonella* isolates using genus-specific primers (139F and 141R) that target the *Salmonella* invasive (*invA*) gene as previously described by Marlony et al., (2003).

2.7. Serotyping

From a total of 263 *Salmonella* isolates recovered and confirmed as *Salmonella* spp, 39 representative isolates were systematically selected to represent various antimicrobial resistance and rep-PCR (BOX-A1R, ERIC and GTG-5) profiles (data not shown), sources and sample type. Isolates were serotyped at the Onderstepoort Veterinary Institute (OVI) Bacteriology Laboratory, Pretoria by slide agglutination test using commercial antisera (BioRad PLC, Davies, PLC). Serovars were identified according to the antigenic formula of the Kauffmann-White Scheme (Grimont & Weill, 2007)

2.8. Statistical analysis

Analyses on *Salmonella* prevalence and serovar frequency and distribution were done using commercially available software (SPSS 17.0; SPSS Inc., Chicago, IL). Descriptive analysis data were reported as either percentage prevalence of *Salmonella* spp. in samples or the diversity of various *Salmonella* serovars from samples that tested positive. Samples were also grouped by their different sites and sources within respective sites in the analyses.

3. Results

3.1. *Salmonella* prevalence

The numbers and percentages of *Salmonella*-positive samples (by sampling site and sample type) are shown in Table 1. Of the 491 samples analysed in this study, 26 (5.3%) were *Salmonella* positive. *Salmonella* was detected at all three sampling sites. Samples from site A had the lowest rate (3.2%; 6/190) of *Salmonella* contamination compared to samples from site B (6.8%; 14/206) and site C (6.3%; 6/95). When considering sample types across regions, the highest proportion of positive samples was recorded on surface samples (7.1%; 9/126) compared to fruit (4.9%; 11/225) and water (5%; 6/140) samples. It is worth noting that only conveyer belt surfaces from one site (site B) tested positive as was also the case with packhouse water which tested positive only for site A. *Salmonella* was not detected in any preharvest samples (both fruit and water) from site A while the opposite was true for the

other two sites (site B and C). No *Salmonella* was recovered from employee (packers and sorters) hands, unwashed packhouse fruit, river, warm bath and tap water for all three sampling sites.

Table 1 *Salmonella* positive samples in the fruit supply chain from the field to the packed product

Sample type	Number of positive samples/total number of samples (%)						Total positive samples	Total isolates recovered
	Site A		Site B		Site C			
	Prevalence	(N)	Prevalence	(N)	Prevalence	(N)		
Orchard fruit	0/60 (0)	0	2/60 (3.3)	20	4/30 (13.3)	35	6/150 (4)	55
Fruit before wash	0/6 (0)	0	0/6 (0)	0	0/3 (0)	0	0/15 (0)	0
Fruit after wash	1/6 (16.7)	16	0/6 (0)	0	0/3 (0)	0	1/15 (6.7)	16
Fruit after warm bath	1/6 (16.7)	25	0/6 (0)	0	0/3 (0)	0	1/15 (6.7)	25
Fruit after waxing	1/6 (16.7)	11	0/6 (0)	0	0/3 (0)	0	1/15 (6.7)	11
Final pack fruit	1/6 (16.7)	11	0/6 (0)	0	1/3 (33.3)	14	2/15 (13.3)	25
River water	0/10 (0)	0	0/10 (0)	0	0/5 (0)	0	0/25 (0)	0
Storage dam water	NA	NA	0/10 (0)	0	1/5 (20)	23	1/15 (6.7)	23
Orchard water	0/10 (0)	0	3/10 (30)	33	0/5 (0)	0	3/25 (12)	33
First wash water	2/10 (50)	21	0/10 (0)	0	0/5 (0)	0	2/25 (8)	21
Warm bath water	0/10 (0)	0	0/10 (0)	0	0/5 (0)	0	0/25 (0)	0
Tap water	0/10 (0)	0	0/10 (0)	0	0/5 (0)	0	0/25 (0)	0
Conveyer belt surfaces	0/25 (0)	0	9/36 (25)	54	0/20 (0)	0	9/81 (11.1)	54
Hand surfaces	0/25 (0)	0	0/20 (0)	0	NA	0	0/45 (0)	0
Total	6/190 (3.2)	84	14/206 (6.8)	107	6/95 (6.3)	72	26/491 (5.3)	263

^b (N) Number of isolates recovered.

3.2. *Salmonella* Serovars

A total of 263 isolates were recovered from the 26 positive samples and identified using MALDI-TOF MS to genus level as *Salmonella* spp. with score values ranging from 2.292 to 2.537. All the 263 *Salmonella* isolates tested positive for the presence of the *invA* gene, as indicated by a single 284 bp of amplified DNA fragment. A total of 39 representative isolates were systematically selected based on their sampling site and sample type and, antimicrobial resistance phenotype and rep-PCR (BOX-A1R, ERIC and GTG-5) profiles, results of which will be reported elsewhere.

Out of the 39 representative isolates serotyped, five different serovars were identified (Table 2). The most commonly isolated serovars were *S. Muenchen* (13/39; 33.3%), followed by *S. Typhimurium* (12/39; 30.8%), *S. Heidelberg* (8/39; 20.5%). *S. Bsilla* (3/39; 7.7%) and *Salm IIb 17: r: z* (2/39; 5.1%). One isolate recovered from site B orchard water was confirmed on

biochemical tests as *Salmonella* spp. However, the strain was a rough biotype that could not be typed.

Across sites, *Salmonella* Typhimurium was isolated most frequently from packhouse fruit but was not isolated from any irrigation water samples. *Salmonella* Heidelberg and *S. Muenchen* were detected the most among the different samples with the former not being detected on any conveyer belts while the later was only absent in packhouse water samples. Serovar Muenchen was the most prevalent serovar (43.8%; 7/16) in preharvest water and fruit samples followed by *S. Heidelberg* (25%; 4/16) and *S. Typhimurium* (6.25%; 1/16). In contrast, *S. Typhimurium* was the most common serovar isolated from postharvest water and fruit samples (47.8%; 11/23) compared to *S. Muenchen* (26.1%; 6/23) and *S. Heidelberg* (17.4%; 4/23). Most samples had multiple serovars with site B orchard water recording the greatest serovar diversity (four serovars).

Table 2 Serovar distribution and diversity of 39 *Salmonella* representative isolates recovered from irrigation water, fruit and packline surfaces

Sample type	Site A		Site B		Site C	
	Serovar (s)	n	Serovar(s)	n	Serovar (s)	n
Irrigation water						
Dam water	-	-	-	-	Heidelberg	1
Orchard water	-	-	Heidelberg	1	-	-
			Muenchen	3	-	-
			Rough	1	-	-
			Salm IIb:17:r:z	2	-	-
Orchard fruit	-	-	Heidelberg	2	Muenchen	4
			Typhimurium	1	S. Bsilla	1
Packhouse water						
First wash water	Typhimurium	2	-	-	-	-
	Heidelberg-	1	-	-	-	-
Packhouse fruit						
Final packed fruit	Typhimurium	8	-	-	Muenchen	4
	Heidelberg	3	-	-	Typhimurium	1
Surfaces (Conveyer belt)	-	-	Muenchen	2	-	-
	-	-	S. Bsilla	2	-	-
Total	Typhimurium	10	Heidelberg	3	Muenchen	8
	Heidelberg	4	Muenchen	5	Typhimurium	1
			Typhimurium	1	S. Bsilla	1
			S. Bsilla	2	Heidelberg	1
			Salm IIb:17:r:z	2		
			Rough	1		

Salmonella serovar diversity was highest for isolates from site B than the other two sites, with all five serovars including the untypable strain, present at this site in similar proportions. In the site C data set, *S. Muenchen* (8 isolates) and serovars Typhimurium, Heidelberg and Bsilla (1 isolate each) were identified. Site A samples had the lowest serovar diversity with *S. Typhimurium* (10 isolates) and *S. Heidelberg* (4 isolates) only, being detected. *Salmonella* serovars Typhimurium and Heidelberg were isolated in all three regions while Salm IIb: 17: r: z was only found in site B orchard water sample. *Salmonella* serovars Muenchen and Bsilla were only isolated from site B and site C and not from site A samples.

4. Discussion

To the best of our knowledge, this is the first extensive report on the prevalence of *Salmonella* in primary production (field) and processing stages (packhouse) in South Africa. The overall prevalence rate (5.3%) observed in the present study is higher than what has been generally reported from previous studies in the developed world (Duffy et al., 2005; Castillo et al., 2004; Giusti et al., 2010; Gorski et al., 2011; Johnston et al., 2005; Micallef et al., 2012., Mukherjee et al., 2006), but comparatively lower than that reported in some other developing countries (Abakpa et al., 2015; Ndiaye et al., 2011; Uyttendaele et al., 2014). According to a study by Uyttendaele et al. (2014), good agricultural practices (GAP) are well understood and implemented in developed countries but not so in developing countries. However, in this study all three farms sampled have been GlobalGAP certified for more than five years while the packhouses are certified to the British Retail Consortium (BRC) standard. Nevertheless, the detection of *Salmonella* at these farms may suggest poor implementation of food safety standards in the production system.

Globally, agricultural water has been recognised as one of the most important potential preharvest source of produce contamination by foodborne pathogens (Gemmell & Schmidt, 2012; Ijabadeniyi et al., 2012; Pachepsky et al., 2011). Contamination of irrigation water with *Salmonella* typically relates to manure use and/or sewage spillages/runoffs. It is important to note that no manure is used in the horticultural production systems assessed in this study and no intensive animal production was evident near any of the sampling sites. Although the quality water used on these farms is at times compromised due to nearby informal settlements or municipal waste water (De Villiers, 2007; Fatoki et al., 2001; CSIR, 2010), *Salmonella* was not detected in any river water samples at the time of sampling. However, one irrigation water sample from a storage dam at site C and three orchard water samples from site B were

found to be *Salmonella* positive. Previous studies have reportedly recovered *Salmonella* from irrigation water and sediments hence the recognition of irrigation water as an important source of *Salmonella* contamination (Duffy et al., 2005; Gallegos-Robles et al., 2008; Izumi et al., 2008; Micallef et al., 2012;).

It is worth noting that the two sites which contained *Salmonella* positive irrigation water samples in our study also recorded *Salmonella* positive orchard fruit samples and it is most likely a result of contamination from the irrigation water at least at site B. The irrigation water from site B was collected from orchard irrigation pipes and although it tested positive for *Salmonella*, the supplying dam water was *Salmonella* negative. It is possible that biofilms in the irrigation pipes can serve as a source of *Salmonella* inoculum, and further investigation is needed to evaluate the survival and persistence of *Salmonella* in irrigation pipe biofilms (Sha et al., 2013).

At site A, *Salmonella* was isolated from packhouse wash water (chlorine spray) and fruit samples including fruit collected immediately after the chlorine spray, after warm bath, after waxing, and final packing. *Salmonella* was not detected from preharvest samples (orchard fruit and irrigation water sources) at site A, while the pathogen was detected in five preharvest samples at sites B and C. It is therefore possible that the fruit contamination observed at site A occurred during processing. Produce contamination during washing can contribute to pathogen transmission to other products during subsequent handling steps (Beuchat, 1998).

At site C, *Salmonella* was only isolated from fruit in the final packing stages and not on any other point in the packhouse. Similarly at site B, *Salmonella* was isolated from conveyer belt surface, but was not detected on any fruit samples that passed through these contact surfaces confirming the sporadic nature of the pathogen in the fresh produce chain (Buck et al., 2003). In a study by Duffy et al. (2005) *Salmonella* was not detected from packing shed fruit despite the presence of the pathogen on packhouse contact surfaces. Other researchers have also reported *Salmonella* on packhouse contact surfaces including conveyer belts, conveyer rollers and unloading ramps similar to our findings (Avila-Vega et al., 2014; Soto-Beltran et al., 2015).

In this study *Salmonella* was not detected on the surfaces of employee hands or gloves, similar to the results reported by Duffy et al. (2005). The same authors stated that absence of *Salmonella* on employee hands does not necessarily cancel out hands as a potential vehicle of transmission of pathogens to the end product given that fresh produce passes through a lot of hands both during harvesting and postharvest handling stages. However, other studies have identified harvesting and packers personnel hands as a potential source of *Salmonella* contamination in cantaloupe and bell pepper production (Materon et al., 2007; Soto-Beltran, 2015).

It is worth noting that none of our river water samples tested positive for *Salmonella*, especially considering previous studies have demonstrated that river water can serve as a reservoir of *Salmonella* (Gorski et al., 2011; Maurer et al., 2015; Pachepsky et al., 2011). This variation can be due to differences in sampling methods, sites and/or length of sampling time. The majority of samples analysed by Gorski et al. (2011) were from publicly accessible water sources collected over a two year study resulting in a 7.1% *Salmonella* prevalence. Surface water samples obtained from the Suwannee River over six months yielded a 96% *Salmonella* prevalence with downstream sites recording higher pathogen concentrations than upstream sites (Rajabi et al., 2011). The downstream sites were in direct contact with human populations whereas the upstream site was in close proximity to the river source and far from human and agriculture activities. Our findings however, do agree with those of Duffy et al. (2005) who also did not detect *Salmonella* from any river water samples collected from the Rio Grande twice in one season.

All three sampling sites processed fruit intended for consumption as fresh fruit destined for both local and export markets. According to the proposed microbiological specifications set out in the Guidelines for Environmental Health Officers in South Africa (DoH, 2000), *Salmonella* should be absent in every 25 g of sample tested, hence the detection of this pathogen in the final product was undesirable. While *Salmonella* was detected on fruit and some environmental samples, to the best of our knowledge, no case of foodborne illness linked to the consumption of fresh produce have been reported in South Africa. However the detection of *Salmonella* in the processing environment emphasises the importance of improved packhouse sanitation conditions to prevent the transfer of this pathogen to the final product.

Our findings on serovar diversity are in agreement with previous reports of low serovar diversity in fresh produce at farm level. Micallef et al. (2012) identified six *Salmonella* serovars (Braenderup, Javiana, Newport, Typhimurium, Tennessee, and Lille) from 63 isolates recovered from irrigation water, sediment and soil on mid-Atlantic tomato farms. Similarly, Duffy et al. (2005) identified seven *Salmonella* serovars (Anatum, Arizona, Javiana, Muenchen, Newport, Rubislaw, and Texas) from 25 isolates obtained from irrigation water, packing shed equipment and cantaloupes in Texas. Soto-Beltran et al. (2015) identified *S. Typhimurium* only out of 50 isolates recovered from bell peppers from the field and packhouse environment in Mexico. Most of the isolates in this study were recovered from processing environments (packhouse), where the settings are relatively confined and have limited pathogen dispersal in comparison to more open environments such as the production environment (field) similar to previous findings (Jokinen et al., 2015; Maurer et al., 2015).

High serovar diversity was detected in site B isolates than the other two sites, with irrigation water contributing to the majority (four serovars) of the isolates suggesting a large variety of host species or sources that can act as reservoirs of *Salmonella* in this area. This site also recorded the highest *Salmonella* prevalence rate followed by sites C and A, implying differences in pathogen loading and contamination (Maurer et al., 2015). Differences in levels of persistence of different serovars in different environments and the ability of different serovars to survive in different environments cannot be ruled out (Gorski et al., 2011; Jokinen et al., 2015; Maurer et al., 2015).

Four of the serovars detected in the present study (Typhimurium, Muenchen, Heidelberg and Bsilla) belong to *Salmonella enterica* subspecies *enterica*, which is responsible for human salmonellosis (CDC, 2011). Furthermore, the occurrence of serovars Typhimurium, Heidelberg and Muenchen in the present study is of high public health significance given that these serovars are listed among the top ten serovars causing human salmonellosis in the United States (CDC, 2013). Additionally, serovars Muenchen and Typhimurium have been previously implicated in outbreaks of *Salmonella* infection linked to fresh produce (Proctor, Hamacher, Tortorello, Archer, & Davis, 2001).

Salmonella Heidelberg is predominantly associated with poultry (Foley et al., 2011), its occurrence in the present study is interesting since no intensive animal production activities were observed in the areas sampled suggesting other hosts such as wildlife as carriers of the

pathogen (Botti et al., 2013; Farias et al., 2014). *Salmonella* Bsilla is a rarely found serovar and we are not aware of any outbreak report related to this organism in the literature. The serovar I1b: 17: r: z is a member of the *Salmonella* enterica subspecies *salamae* which is a rare but clinically important subspecies mostly isolated from reptiles (Sharon, Ni and Janda, 2012) suggesting that these reservoirs might have played a role in irrigation water contamination in cases where the serovar was isolated.

Although there was serovar overlap among samples from site A (packhouse water and packhouse fruit), site B (irrigation water and orchard fruit) and site C (orchard fruit and packhouse fruit), we could not make conclusions on the possible serovar transfer between sampling points. Subtyping methods with higher discriminatory power are needed to determine if serotypes appearing in more than one sample from the same location are genotypically related. As previously mentioned in this report, all isolates were further analysed using other techniques; antimicrobial resistance profiling, three rep-PCR primers (BOX A1R, ERIC and GTG₅) and MALDI-TOF-MS; results will be reported elsewhere. Using PFGE analysis, Duffy et al. (2005) reported that *Salmonella* isolates recovered from irrigation water were not genetically related to those found on fresh produce. Similarly, Gorski et al. (2011) reported a lack of genetic relatedness among water and wildlife *Salmonella* serotypes since water sample serotypes did not cluster together with any of the wildlife sample serotypes. Nonetheless, the researchers observed some near matches implying a potential link between water and wildlife contamination. However this aspect needs to be further investigated in crop agricultural settings and its link to public health.

All isolates assayed in this study harboured the virulence gene (*invA*) indicating that they were all potentially pathogenic. Other studies have also reported high prevalence rates of this gene in *Salmonella* spp. (Bisi-Johnson et al., 2011). The *invA* gene is critical for full virulence in *Salmonella* as it encodes for a protein in the inner membrane of the bacterium necessary for the invasion of deeper epithelial host cells (Khan, Nawaz, Khan & Cerniglia, 1999). Our findings further support the suitability of the *invA* gene as an international standard procedure for detection and confirmation of *Salmonella* genus both in food and environmental samples (Malorny et al., 2003).

5. Conclusion

This study provides baseline information regarding the prevalence and serovar diversity of *Salmonella* in fresh produce environments. Although we were able to identify potential sources of *Salmonella* contamination in some environments overall prevalence and serovar diversity were low. Analysis of *Salmonella* serovar diversity from production through to packing is critical in identifying the transmission routes and areas that need more attention for mitigation strategies. This study further confirms the importance of fresh produce and their production environments as potential reservoirs and carriers of clinically significant *Salmonella* serovars. Further studies should consider determining factors affecting *Salmonella* prevalence rates in different regions and transfer between environmental systems and its link with food safety concerns.

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References

- Abakpa, G. O., Umoh, V. J., Ameh, J. B., Yakubu, S. E., Kwaga, J. K. P., & Kamaruzaman, S. (2015). Diversity and antimicrobial resistance of *Salmonella* enterica isolated from fresh produce and environmental samples. *Environmental Nanotechnology, Monitoring and Management* 3 (2015): 38–46
- Andrews, W. H., & Hammack, T. (2007). *Salmonella*. In Bacteriological Analytical Manual, Chapter 5. December 2007 Edition. U. S. Food and Drug Administration.
- Avila-Vega, D. E., Alvarez-Mayorga, B., Arvizu-Medrano, S. M., Pacheco-Aguilar, R., Martinez-Peniche, R., & Hernandez-Iturriaga, M. (2014). Microbiological profile and incidence of *Salmonella* and *Listeria monocytogenes* on hydroponic bell peppers and greenhouse cultivation environment. *Journal of Food Protection*, 77 (11), 1904-1910.
- Beuchat, L. R., & Ryu, J. H. (1997). Produce handling and processing practices. *Emerging Infectious Diseases*, 3(4), 459.

- Bisi-Johnson, M. A., Obi, C. L., Vasaikar, S. D., Baba, K. A., & Hattori, T. (2011). Molecular basis of virulence in clinical isolates of *Escherichia coli* and *Salmonella* species from a tertiary hospital in the Eastern Cape, South Africa. *Gut Pathogens* 3(9).
- Botti, V., Navillod, F. V., Domenis, L., Orusa, R., Pepe, E., Robetto, S., & Guidetti, C. (2013). *Salmonella* spp. and antibiotic-resistant strains in wild mammals and birds in north-western Italy from 2002 to 2010. *Vet. Ital*, 49(2), 195-202.
- Buck, J. W., Walcott, R. R., & Beuchat, L. R. (2003). Recent trends in microbiological safety of fruits and vegetables. Online. Plant Health Progress. doi: 10.1094/PHP-2003-0121-01-RV.
- Callejon, R. M., Rodriguez-Naranjo, M. I., Ubeda, C., Hornedo-Ortega, R., Garcia-Parrilla, M. C., & Troncoso, A. M. (2015). Reported foodborne outbreaks due to fresh produce in the United States and European Union: trends and causes. *Foodborne Pathogen and Disease*, 12(1):32–8. doi: 10.1089/fpd.2014.1821
- Castillo, A., Villarruel-Lopez, A., Navarro-Hidalgo, V., Martinez-Gonzalez, N. E., & Torres-Vitela, M. R. (2004). *Salmonella* contamination during production of cantaloupe: a binational study. *Journal of Food Protection*, 67, 713–720.
- Centers for Disease Control and Prevention (CDC). (2011). National *Salmonella* Surveillance Overview. Atlanta, Georgia: US Department of Health and Human Services, CDC. Accessed on 21 November 2015.
- Centers for Disease Control and Prevention (CDC). (2013). National *Salmonella* Surveillance Annual Report, 2011. Atlanta, Georgia: US Department of Health and Human Services.
- Department of Health (DoH). (2000). Guidelines for environmental health officers on the interpretation of microbiological analysis data of food. South Africa: Department of Health Directorate: Food Control.
- Duffy, E. A., Lucia, L. M., Kells, J. M., Castillo, A., Pillai, S. D., & Acuff, G. R. (2005). Concentrations of *Escherichia coli* and genetic diversity and antibiotic resistance profiling of *Salmonella* isolated from irrigation water, packing shed equipment, and fresh produce in Texas. *Journal of Food Protection*, 68, 70–79.
- Farias, L. F. P., Oliveira, C. J. B., Medardus, J. J., Molla, B. Z., Wolfe, B. A., & Gebreyes, W. A. (2014). Phenotypic and genotypic characterization of *Salmonella enterica* in captive wildlife and exotic animal species in Ohio, USA. *Zoonoses and Public Health*.
- Foley, S. L., Nayak, R., Hanning, I. B., Johnson, T. J., Han, J., & Ricke, S. C. (2011). Population dynamics of *Salmonella enterica* serotypes in commercial egg and poultry production. Minireview. *Applied and Environmental Microbiology*, 77(13), 4273–4279.

- Gallegos-Robles, M., Loreda, A., Ojeda, G., Vega, A., Chew, Y., Velarde, S., & Fratamico, P. (2008). Identification of *Salmonella* Serotypes isolated from cantaloupe and chile pepper production systems in Mexico by PCR-Restriction Fragment Length Polymorphism. *Journal of Food Protection*, 71(11), 2217-2222.
- Gemmell, M. E., & Schmidt, S. (2012). Microbiological assessment of river water used for the irrigation of fresh produce in a sub-urban community in Sobantu, South Africa. *Food Research International*, 47, 300-305.
- Giusti, M., Aurigemma, C., Marinelli, L., Tufi, D., De Medici, D., Di Pasquale, S.,...Boccia, A. (2010). The evaluation of the microbial safety of fresh ready-to-eat vegetables produced by different technologies in Italy. *Journal of Applied Microbiology*, 109, 996-1006.
- Gorski, L., Parker, C. T., Liang, A., Cooley, M. B., Jay-Russell, M. T., Gordus, A. G.,...Mandrell, R. E. (2011). Prevalence, distribution, and diversity of *Salmonella enterica* in a major produce region of California. *Applied and Environmental Microbiology*, 77(8), 2734-48.
- Grimont, P. A. D., & Weill, F. X. (2007). Antigenic formulae of the *Salmonella* serovars (9th Ed.). Paris, France: Institute Pasteur. World Health Organization Collaborating Center for Reference and Research on *Salmonella*.
- Hendriksen, R. S. (2003). Global Salm-Surv (A global *Salmonella* surveillance and laboratory support project of the World Health Organization). *Laboratory Protocols, Level, 1*, 1-19.
- Ijabadeniyi, O. A., Debusho, L. K., van der Linde, M., & Buys, E.M. (2012). Irrigation water as a potential pre-harvest source of bacterial contamination of vegetables. *Journal of Food Safety*, 31, 452-461.
- Isaacs, S., Aramini, J., Ciebin, B., Farrar, J. A., Ahmed, R., Middleton, D., ... & *Salmonella* Enteritidis PT30 Outbreak Investigation Working Group. (2005). An international outbreak of salmonellosis associated with raw almonds contaminated with a rare phage type of *Salmonella* Enteritidis. *Journal of Food Protection*, 68(1), 191-198.
- Jackson, B. R., Griffin, P. M., Cole, D., Walsh, K. A., & Chai, S. J. (2013). Outbreak-associated *Salmonella* enterica serotypes and food commodities, United States, 1998–2008. *Emerging infectious diseases*, 19(8), 1239.
- Johnston, L. M., Jaykus, L. A., Moll, D., Martinez, M. C., Anciso, J., Mora, B.,...Moe, C. L. (2005). A field study of the microbiological quality of fresh produce. *Journal of Food Protection*, 68, 1840-1847.

- Jokinen, C. C., Koot, J., Cole, L., Desruisseau, A., Edge, T. A., Khan, I. U. H.,...Gannon V.P.J. (2015). The distribution of *Salmonella* enterica serovars and subtypes in surface water from five agricultural regions across Canada. *Water Research*, 76, 120-131.
- Khan, A. A., Nawaz, M. S., Khan, S. A & Cerniglia, C. E. (1999). Detection of multidrug resistant *Salmonella* Typhimurium DT104 by multiplex polymerase chain reaction. *FEMS Microbiology Letter*, 182, 355-360.
- Ktsoyan, Z., Ghazaryan, K., Manukyan, G., Martirosyan, A., Mnatsakanyan, A., Arakelova, K.,...Amino, R. (2013). Inflammatory responses to *Salmonella* infections are serotype-specific. *International Journal of Bacteriology*. doi:10.1155/2013/168179.
- Langholz, J. A., & Jay-Russell, M. T. (2013). Potential role of wildlife in pathogenic contamination of fresh produce. *Human-Wildlife Interactions*, 7(1), 140-57.
- Lopez-Velasco, G., Tomas-Callejas, A., Diribsa, D., Wei, P. & Suslow, T.V. (2013). Growth of *Salmonella* enterica in foliar pesticide solutions and its survival during field production and postharvest handling of fresh market tomato. *Journal of Applied Microbiology*, 114, 1547-558.
- Materon, L. A., Martinez-Garcia, M., & McDonald, V. (2007). Identification of sources of microbial pathogens on cantaloupe rinds from pre-harvest to post-harvest operations. *World Journal Microbiology and Biotechnology*, 23, 1281-1287.
- Maurer, J. J., Martin, G., Hernandez, S., Cheng, Y, Gerner-Smidt, P., Hise, K. B., ... & Lipp, E. K. (2015). Diversity and persistence of *Salmonella* enterica strains in rural landscapes in the Southeastern United States. *PloS one*, 10(7).
- Micallef, S. A., Goldstein, R. E. R., George, A., Kleinfelter, L., Boyer, M. S., McLaughlin, C. R.,...Sapkota, A. R. (2012). Occurrence and antibiotic resistance of multiple *Salmonella* serotypes recovered from water, sediment, and soil on mid-Atlantic tomato farms. *Environmental Research*, 114:31–39.
- Mukherjee, A., Speh, D., Jones, A.T., Buesing, K.M., & Diez-Gonzalez, F. (2006). Longitudinal microbiological survey of fresh produce grown by farmers in the upper Midwest. *Journal of Food Protection*, 69, 1928–1936.
- Ndiaye, M. L., Dieng, Y., Niang, S., Pfeifer, H. R, Tonolla, M., & Peduzzi, R. (2011). Effect of irrigation water on the incidence of *Salmonella* spp. on lettuce produced by urban agriculture and sold in markets in Dakar Senegal. *African Journal of Microbiological Research*, 5 (19), 2885–2890

- Ng, P. J., Fleet, G. H., & Heard, G. M. (2005). Pesticides as a source of microbial contamination of salad vegetables. *International Journal of Food Microbiology*, 101: 237–250
- Nielsen, E. M., Skov, M. N., Madsen, J. J., Lodal, J., Jespersen, J. B., & Baggesen, D. L. (2004). Verocytotoxin-producing *Escherichia coli* in wild birds and rodents in close proximity to farms. *Applied and Environmental Microbiology*, 70(11), 6944-6947.
- Pachepsky, Y., Shelton, D. R., McLain, J. E. T., Patel, J., & Mandrell, R. E. (2011). Irrigation waters as a source of pathogenic microorganisms in produce: A Review. *Advances in Agronomy*, 113: 73-138.
- Painter, J. A., Hoekstra, R. M., Ayers, T., Tauxe, R. V., Braden, C. R., Angulo, F. J., & Griffin, P. M. (2013). Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998–2008. *Emerging Infectious Diseases*, 19 (3), 407-415.
- Proctor, M. E., Hamacher, M., Tortorello, M. L., Archer, J. R., & Davis, J. P. (2001). Multistate outbreak of *Salmonella* serovar Muenchen infections associated with alfalfa sprouts grown from seeds pre-treated with calcium hypochlorite. *Journal of clinical microbiology*, 39(10), 3461-3465.
- Rajabi, M., Jones, M., Hubbard, M., Rodrick, G., & Wright, A. C. (2011). Distribution and genetic diversity of *Salmonella enterica* in the Upper Suwannee River. *International Journal of Microbiology*. doi:10.1155/2011/461321
- Sha, Q., Forstner, M. R., & Hahn, D. (2013). Diversity of *Salmonella* in biofilms and water in a headwater ecosystem. *FEMS Microbiology Ecology*, 83 (3), 642-649.
- Sharon, L. A., Ni, F. C.Y., & Janda, J. M. (2012). Increase in extra-intestinal infections caused by *Salmonella enterica* subspecies II–IV. *Emerging Infectious Diseases*, 18 (4), 637-639
- Sivapalasingam, S., Barrett, E., Kimura, A., Van Duyne, S., De Witt, W., Ying, M., ... & Slutsker, L. (2003). A multistate outbreak of *Salmonella enterica* Serotype Newport infection linked to mango consumption: impact of water-dip disinfestation technology. *Clinical Infectious Diseases*, 37(12), 1585-1590.
- Sivapalasingam, S., Friedman, C. R., Cohen, L., & Tauxe, R. V. (2004). Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *Journal of Food Protection*, 67(10), 2342-2353.

- Soto-Beltran, M., Campo, C. N., Campos-Sauceda, J., Aven-Bustillos, R., & Chaidez, C. (2015). Prevalence of *Salmonella*, *Escherichia coli* and coliforms on bell pepper from the field to the packing process. *African Journal of Microbiology Research*, 9(10), 718-724.
- Strawn, L. K., Gröh, Y. T., Warchocki, S., Worobo, R. W., Bihn, E. A., & Wiedmann, M. (2013). Risk factors associated with *Salmonella* and *Listeria monocytogenes* contamination of produce fields. *Applied and Environmental Microbiology*, 79 (24), 7618–7627.
- Uesugi, A. R., Danyluk, M. D., Mandrell, R. E., & Harris, L. J. (2007). Isolation of *Salmonella* Enteritidis phage type 30 from a single almond orchard over a 5-year period. *Journal of Food Protection*, 70(8), 1784-1789.
- Uyttendaele, M., Moneim, A. A., Ceuppens, S., & Tahan, F. E. (2014). Microbiological safety of strawberries and lettuce for domestic consumption in Egypt. *Journal of Food Processing and Technology*, 5, 308. doi:10.4172/2157-7110.1000308.
- Waldner, L. L., MacKenzie, K. D., Köster, W., & White, A. P. (2012). From Exit to Entry: Long-term survival and transmission of *Salmonella*. *Pathogens* 1, 128-155: doi:10.3390/pathogens1020128