Biofilm formation of *Salmonella* species isolated from fresh cabbage and spinach

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ABSTRACT: The aim of the study was to isolate *Salmonella* from fresh cabbage and spinach vegetables, determine antimicrobial resistance and biofilm formation of the isolates. Spinach and cabbage farm vegetables were found to harbour *Salmonella*. A total of eighty-two *Salmonella* isolates were recovered from both vegetables and subjected to antimicrobial reactions. *Salmonella* isolate showed sensitivity against the aminoglycoside and quinolones. Isolates from cabbage showed $\geq 80\%$ susceptibility to nalidixic acid and ciprofloxacin and an average of 72% susceptibility was exhibited against gentamicin and ofloxacin. Also, isolates from spinach vegetable demonstrated excellent sensitivity against chloramphenicol (94%), nalidixic acid (90%) and ofloxacin (82%). Variable resistant patterns was observed for tetracycline (58%: 47%), ampicillin (55.5%: 31.4%), erythromycin (58.1%: 62.7%), streptomycin (64.5%: 76.5%), cephalothin (35.5%: 39.2%) against isolates from cabbage and spinach respectively. The assessment of biofilm formation by *Salmonella* on microtitre plate showed that all *Salmonella* isolates were able to form biofilms. Isolates from cabbage were mainly strong producer 15(48.3%), while 11(35.5%) of the isolates were moderate producers and 6(16.1%) weak producer. On the other hand, 28(54.9%) of *Salmonella* isolates from spinach vegetable were moderate producer, 12(23.5%) weak producer and 11(21.5%) strong producer. The finding of this study shows that cabbage and spinach is potential host for the transmission of *Salmonella* to humans or other animals. The ability of the isolates to form biofilm reveals the potential of the isolates to persist on the vegetable and the pathogenic status of the isolates as well as ability to resist antimicrobial chemotherapy. © JASEM

Introduction
Fresh vegetables are fundamental components of the human diet and there is considerable evidence of the health and nutritional benefits associated with the consumption of fresh vegetables. This has led to significant rise in the demand of fresh produce, changes in life styles and major shifts in consumption trends (Abadias *et al.,* 2008; Tang *et al.,* 2012).Vegetables can become contaminated with microorganisms capable of causing human diseases while still on the fields (Mukherjee *et al.,* 2006). Bacteria such as *Clostridium botulinum, Bacillus cereus* and *Listeria monocytogenes* capable of causing disease, are normal inhabitants of many soils, while *Salmonella, Shigella, Escherichia coli* and *Campylobacter* which reside in the intestinal tracts of humans and animals, are more likely to contaminate raw vegetables through contact with faeces, sewage, untreated irrigation water or surface water (Cliver, 1997; Speer, 1997). Fresh farm produce can be a vehicle for the transmission of bacterial, parasitic and viral pathogens capable of causing human illness. There is documented evidence of raw vegetables harbouring potential food-borne pathogens, which can become contaminated while growing or during harvesting, post-harvest, handling, or distribution (Mukherjee *et al.,* 2006; Abadias *et al.,* 2008).

Bacterial pathogens continue to be a major contributor to produce-associated food-borne illnesses (Berger *et al.,* 2010).The incidence and frequency of foodborne outbreaks caused by contaminated fresh vegetables is on the increase (De Roever, 1998; Wegener *et al.,* 2003; Raufu *et al.,* 2014). *Salmonella* is one of the pathogen most frequently linked to consumption of fruit and vegetables (Sivapalasingam *et al.,* 2004). Several outbreaks of salmonellosis have been traced to contaminated fresh fruits and vegetables (Salleh *et al.,* 2003; Berger *et al.,* 2010). A wide spectrum of produce vehicles have been associated with *Salmonella* infections (Berger *et al.,* 2010). The factors influencing the increase in salmonellosis due to vegetables are changes in agricultural practices, eating habits and increases in the worldwide commerce of fresh produce (Collins, 1997; Raufu *et al.,* 2014).

Reports has shown that fresh spinach grown in three Californian countries were the source of microbial contamination that led to food-borne outbreak affecting 26 US states which resulted in about 200

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cases of illness, including some of haemolytic uremic syndrome and resulted in three deaths (FDA, 2006; Abadias *et al.,* 2008).The consumption of fresh fruit and vegetables might therefore pose a food safety risk because they are susceptible to contamination by faecal material on the farm (Mukherjee *et al.,* 2004; Abadias *et al.,* 2008).

In present times, bacterial biofilms have been more linked to food safety issues globally. Biofilm is formed when bacterial cells attach to one another and stick on to a contact surface. Biofilm are hazardous as they can become a persistence source of contamination (Houdt and Michiels, 2010). The existence of pathogenic organisms in biofilms has been linked to foodborne illness outbreaks in cantaloupe melons, apples, and leafy greens (Annous *et al.,* 2009). They are capable of adhering to plant surfaces and forcefully infect the plants interior (Schikora *et al*., 2012). Bacteria living within biofilms can exhibit 1000 times more resistance to antimicrobials than their planktonic peers. The close proximity of these bacteria within biofilm community enhances gene transfer; resulting in increased genetic diversity of antimicrobial resistance. Once biofilm forms on fresh produce surface, they not only can cause cross contamination to other food produce or processing equipment surfaces in industry, they also result in a potent health hazard to consumers (Tang *et al.,* 2012).

As food is largely the vehicle for the transmission of *Salmonella* to humans it is desirable that all data on contamination of the various food types are available for analysis (Duggan *et al.,* 2012). As a result of the serious implications from the consumption of contaminated vegetables, this work aimed at investigating *Salmonella* contamination of two major vegetables grown in Alice, Eastern Cape Province of South Africa, to determine their antibiotic resistance pattern as well as the adherence and pathogenic status of *Salmonella.*

MATERIALS AND METHODS

Sample collection: Twenty samples of fresh farm vegetables were collected from a vegetable farm in Eastern Cape Province of South Africa. Samples were kept in separate sterile plastic bags, and placed in a cooler with frozen gel packs and transport to the laboratory on the same day of collection for isolation and identification of *Salmonella.* Ten samples each of spinach and cabbage vegetables were collected.

Sample preparation: The method of Selleh *et al.* (2003) was used for the preparation of samples with modification. Briefly, the vegetables were placed on a working bench in aseptical environment and carefully separated. The separated parts were placed in a stomaching bag containing 225 mL of buffered

peptone water (BPW) and incubated at 37 °C overnight.

Salmonella isolation and identification: One hundred millilitres of the pre-enriched samples was inoculated into 10 mL of Rappaport Vassiliadis Broth, (Merck) and 9 ml of Mannitol Selenite Cystine Broth (Oxoid) and incubated for 24 h at 42 °C and 37 °C respectively. A loopful from each of the enriched broths were streaked onto Hektoen Enteric Agar (HEK) (Merck), Salmonella-Shigella Agar (Merck) and Xylose Lysine Deoxycholate Agar (XLD) (Merck), Agar plates were incubated at 37 °C for 18 to 24 h. About 5 characteristic colonies of *Salmonella* were randomly picked from each plate and inoculated into Triple Sugar Iron Agar (TSA) (Merck) and Lysine Iron Agar (LIA) (Merck) slants. Each culture showing presumptive-positive TSI and LIA results were maintained on Tryptone Soya Agar (TSA)(Merck) (Salleh *et al.,* 2003; Lertworapreecha *et al.,* 2013).Gram reaction and oxidase test were carried out on the presumptive isolates. Suspected *Salmonella* colonies were confirmed by biochemical reactions using Analytical Profile Index (API) 20E strips (bioMérieux, Marcy-L'Étoile France).

Antimicrobial Susceptibility Testing: All isolates were tested for 12 antimicrobial drugs. Antibiotic disks were purchased from Mast Diagnostics (Mast Group Merseyside UK). The antibiotics used are as listed in Table 1. Isolates were sub-cultured on nutrient agar plates incubated for 24 h at 37 °C. Colonies were picked from the agar plates, and suspended in normal saline (0.85% w/v), and the density of the suspension was adjusted to 0.5 McFarland standard. The bacterialsuspension was spread on the Mueller Hinton agar plates using a sterile swab stick, allowed to dry, and impregnated with antibiotic disk (Igbinosa *et al.,* 2013). Plates were incubated at 37 °C for 24 h. Diameters of the zones of inhibition were measured and interpreted, as susceptible, intermediate or resistant according to the Clinical Laboratory Standard Institute guidelines (CLSI 2006).

Biofilm Formation Assay: Quantitatively, biofilm formation among *Salmonella* isolates was assessed using microtitre plate method as described by Igbinosa et al. (2013). Flat bottomed 96 wells microtiter plates were dispensed with 200 μL of Tryptone Soy Broth (TSB) and inoculated with 20 μL of *Salmonella* isolates grown overnight and standardized to 0.5 McFarland standards. Plates were incubated at 37 °C for 24 h. Contents of each well were discarded and washed with sterile phosphate-buffered saline (PBS). Wells were allowed to air dry and stained with 200 μL of 1% crystal violet for 30 min. The wells were carefully washed with distilled water to remove the excess stain. Plates were allowed to dry at room temperature. Dye bound to adherent

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cells was resolubilized with 150 μL of absolute ethanol. Microplate reader (Synergy mx Biotek, USA) was used to read the plates at 570 nm wavelength. Average optical density (OD) of each duplicate result was taken including positive and negative controls. Isolates were categorized as non-biofilm producer (ODi<ODc), weak (ODc<ODi< 0.1), moderate (ODi = 0.1 < 0.12) and strong (ODi> 0.12) producers according to the modified methods of (Cevahir *et al.*, 2008).

Table 1: List of antimicrobial used in the study

Antibiotics code	Antibiotics	Disk content	Antibiotic class
TET	Tetracycline	10 μg	Tetracyclines
CHL	Chloramphenicol	30 μg	Phenicols
AMP	Ampicillin	25 μg	Penicillins
CIP	Ciprofloxacin	5 μg	Fluoroquinolones
GEN	Gentamicin	10 μg	Aminoglycosides
NAL	Nalidixic acid	30 μg	Quinolones
CEP	Cephalothin	30 μg	Cephalosporins (First generation)
STR	Streptomycin	10 μg	Aminoglycosides
TXM	Trimethoprim-sulfamethoxazole	25 μg	Sulfonamides
OFL	Ofloxacin	5 μg	Fluoroquinolones
ERY	Erythromycin	15 μg	Macrolides
VAN	Vancomycin	30 μg	Glycopeptide

RESULTS AND DISCUSSION

Salmonella is an important food-borne pathogen and its prevalence in fresh food poses a threat to human. The increase in demand and consumption of raw vegetables has resulted in a rise in food-borne related illnesses and outbreaks. Fresh vegetable have been reported to anchor potential food-borne pathogens including *Salmonella* (Harris *et al.,* 2003; CDC, 2009).

In the study, spinach and cabbage farm vegetables were found to harbour *Salmonella*. Thirty-one (31) *Salmonella* isolates were isolated from cabbage vegetables while fifty-one (51) *Salmonella* isolates were isolated from spinach vegetables. The incidence and predominance of *Salmonella* in green leafy vegetables including lettuce and cabbage has been documented (Nillian *et al.,* 2011).This is also in agreement with the findings of Chia *et al.* (2007) where leafy vegetables might permit more surface attachment that contribute to the high rate of *Salmonella* survival.These vegetables are top soil creeper hence soil may be a potential source of contamination especially if animal waste have been used as fertilizer (Nillian *et al.,* 2011). Animal waste such as fresh faeces or human faeces from incompletely discomposed sludge from wastewater system when used as fertilizer could result to a primary source of contamination of the farm vegetables. When such fertilizers are applied to the soil, any contact or closest proximity of these farm vegetables to soil leads to contamination of the vegetable. In contrast, water is likely to be an important source of contamination of farm produce. Use of untreated wastewater for irrigation or irrigation water from a contaminated source is a major contributing factor to contamination. Improperly treated wastewater effluent disposed to fresh water system is a contributing source of contaminant to the river, and as such could contaminate farm vegetable when such water body is used as a source of irrigation to farm produce. Research has shown uptake and internalization of *E. coli* O157:H7 in spinach leaves after contaminated water was used for irrigation on the leaves (Mitra *et al.,* 2009; Berger *et al.,* 2010), thereby highlighting the potential risk associated with use of contaminated irrigation water.

During the cultivation stage, pathogenic organisms can establish themselves on growing crops. The risk can be enlarged after harvest either by further direct contamination or by proliferation of existing pathogen populations during processing and post harvest handling activities (Berger *et al.,* 2010). Generally, *Salmonella* infection is self-limiting, however when symptoms persist, antimicrobial therapy is used. Hence the antimicrobial susceptibility of the isolates was carried out. The isolates showed diverse susceptibility profiles against the antibiotics under studied. Multiple antibiotic resistances were found against different classes of antibiotics; all the same, *Salmonella* isolate showed sensitivity against the aminoglycoside and quinolones (Table 2). Isolates from cabbage showed ≥ 80% susceptibility to nalidixic acid and ciprofloxacin while an average of 72% susceptibility was exhibited against gentamicin and ofloxacin. On the other hand, isolates from spinach vegetable demonstrated excellent sensitivity against chloramphenicol (94%), nalidixic acid (90%) and ofloxacin (82%). All *Salmonella* isolates from both sources were absolutely (100%) resistant to vancomycin, 51% resistant to trimethoprim-sulfamethoxazole, whereas variable resistance patterns was observed for tetracycline (58%: 47%), ampicillin (55.5%: 31.4%), erythromycin (58.1% : 62.7%), streptomycin (64.5% : 76.5%), cephalothin (35.5% : 39.2%) against isolates from cabbage and spinach respectively (Table 2). The isolates had a similar trend of

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antibiotic phenotypes suggesting a common source of contamination, however further molecular and epidemiological characterization is required to determine the linkage in source of contamination.

Table 2: Antibiotic susceptibility profile of *Salmonella* isolated from vegetable sources

Antibiotics	Cabbage (*n*=31)			Spinach (*n*=51)		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Tetracycline	5(16.1)	8(25.8)	18(58)	17(33.3)	10(19.6)	24(47)
Chloramphenicol	24(77)	6(19.3)	1(3.2)	48(94.1)	3(5.9)	0(0)
Ampicillin	14(45.2)	0(0)	17(55.5)	30(58.8)	5(9.8)	16(31.4)
Ciprofloxacin	29(93.5)	2(6.5)	0 (0)	38(74.5)	6(11.8)	7(13.7)
Gentamicin	22(71)	9(29.0)	0(0)	40(78.4)	11(21.6)	0(0)
Nalidixic acid	25(80.7)	6(19.4)	0(0)	46(90.2)	2(3.9)	3(5.9)
Cephalothin	13(41.9)	7(22.6)	11(35.5)	11(21.6)	10(19.6)	20(39.2)
Streptomycin	20(64.5)	5(16.1)	6(19.4)	39(76.5)	22(3.9)	10(19.6)
Trimethoprim-sulfamethoxazole	10(32.3)	5(16.1)	16(51.3)	18(35.3)	7(13.7)	26(51)
Ofloxacin	23(74.2)	6(19.4)	2(6.5)	42(82.4)	4(7.8)	5(9.8)
Erythromycin	9(29.0)	4(12.9)	18(58.1)	10(19.6)	9(17.6)	32(62.7)
Vancomycin	0(0)	0(0)	31(100)	0(0)	0(0)	51(100)

Key: S= susceptible, I= intermediate, R=resistance

Several studies have documented high resistance of *salmonella* to the tetracyclines (Yoke-Kqueen *et al.,* 2008; Learn-Han *et al.,* 2009), which is in agreement with the result obtained in this study. The high resistance phenotypes rate of tetracycline observed in the study could be as a result of the use of tetracycline in food animal production which has led to worldwide spread of tetracycline resistance observed in *Salmonella* isolates (White *et al.,* 2001; Logue *et al.,* 2003; Parveen *et al.,* 2007). Thereby indicating longitudinal transfer of resistance genes between *Salmonella* isolates from animal-related sources to vegetable sources (Learn-Han *et al.,* 2009). *Salmonella* isolates from cabbage vegetable were highly sensitive to the quinolones and fluoroquinolones antibiotics compared to isolates from spinach vegetable. The absence of resistance to the fluoroquinolones by *salmonella* serovars from vegetable in Nigeria has been documented (Raufu *et al.,* 2014). *Salmonella* resistance to the fluoroquinolones (ciprofloxacin) is of great concern to public health as invasive forms of salmonellosis are treated with these compounds (Gordon, 2000; White *et al.,* 2001; Learn-Han *et al.,* 2009).

Although variable sensitivity with chloramphenicol was observed against isolates form both sources, however, the low resistance rate might be attributed to the controlled use of chloramphenicol due to the concern of its severe side effects which indirectly increases the efficiency of the antibiotic (Kambal, 1996; Learn-Han *et al.,* 2009).The occurrence of multi-drug resistance *Salmonella* from fresh vegetables is of global health concern as this could led to major healthcare challenge since multi-drug resistance hinder the possibility of therapeutic treatments. The health benefits of consumption of vegetables has led to significant rise of eating of vegetables among the pregnant, young, old, and ill challenged individuals thereby leading to higher risk

of infection among these group of consumers. Hence this is vital in the risk assessment and management of the consumption of vegetables.

A number of studies have shown that *Salmonella* spp are capable of adhering and forming biofilms on diverse surfaces including metal, glass and rubber surfaces (Hood and Zottola, 1997; Joseph *et al.,* 2001; Stepanovic´*et al.,* 2004). The assessment of biofilm formation by *Salmonella* on microtitre plate showed that all *Salmonella* isolates were able to form biofilms. The bacteria under study were able to form biofilm on microtiter plate potentiating its ability to form biofilm on different surfaces. Isolates from cabbage were mainly strong producer 15(48.3%), while 11(35.5%) of the isolates were moderate producers and 6(16.1%) weak producer as shown in Fig 1. On the other hand, 28(54.9%) of *Salmonella* isolates from spinach vegetable were moderate producer, 12(23.5%) weak producer and 11(21.5%) strong producer.

Ability of *Salmonella* to form biofilm even at minimum nutrient medium has been demonstrated (Stepanovic´*et al.,* 2004). Also the efficiency of TSB in *Salmonella* has also been reported. The biofilm formation of the *Salmonella* isolates observed in the study may have been enhanced by favourable growth media, however literature has shown that *Salmonella* is able to form biofilm even at minimal nutrient medium.The study reveals that *Salmonella* isolated from vegetable is able to form biofilms. A correlation between the capacity to produce biofilms and the attachment to leaves, with *Salmonella* showing the efficient adhesion to lettuce leaves has been documented (Patel and Sharma, 2010; Schikora *et al.,* 2012). Hence, the biofilm forming ability demonstrated by these *Salmonella* isolates reveals the pathogenic status of the isolates. Bacteria can use multiple hosts as channel to human or other animals.

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Salmonella is capable of using plant as an alternative route to human; therefore, the consumption of contaminated vegetables poses a health risk to humans.

Management of growing conditions are essential in preventing the contamination of fresh farm produce by human pathogens. Preventive strategies should be employed to avert the contamination of farm vegetables, which include good agricultural practices. Strategies similar to hazard analysis and critical control point programs, which have been effectively used in other areas of food production, if scientifically applied to animal production industries, may improve food safety.

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