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 ≥ 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%), cephaplatin (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

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**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 field (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; Raifu 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; Raifu 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
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 bacterial suspension was spread on the Mueller Hinton agar plates using a sterile swab stick, allowed to dry, and impregnated with antibiotic disk (Iginosa 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 Iginosa 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

*ISOKEN H. IGINOSA
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).

### 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 decomposed 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%), cephalexin (35.5% : 39.2%) against isolates from cabbage and spinach respectively (Table 2). The isolates had a similar trend of

### 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        | Pencillins        |
| CIP              | Ciprofloxacin   | 5 µg         | Fluoroquinolones  |
| GEN              | Gentamicin      | 10 µg        | Aminoglycosides   |
| NAL              | Nalidixic acid  | 30 µg        | Quinolones        |
| CEP              | Cephalothin     | 30 µg        | Cephalosporins    |
| STR              | Streptomycin    | 10 µg        | (First generation)|
| TXM              | Trimethoprim-   | 25 µg        | Sulfonamides      |
|                  | sulfamethoxazole|              |                   |
| OFL              | Ofloxacin       | 5 µg         | Fluoroquinolones  |
| ERV              | Erythromycin    | 15 µg        | Macrolides        |
| VAN              | Vancomycin      | 30 µg        | Glycopeptide      |

*ISOKEN H. IGBINOSA*
Table 2: Antibiotic susceptibility profile of Salmonella isolated from vegetable sources

| Antibiotics          | Cabbage (n=31) | Spinach (n=51) |
|----------------------|----------------|----------------|
| Tetracycline         | S (%) 1(3.2)   | S (%) 7(13.7)  |
| Chloramphenicol      | S (%) 7(22.6)  | S (%) 51(100)  |
| Ampicillin           | S (%) 6(19.4)  | S (%) 0(0)     |
| Ciprofloxacin        | S (%) 2(6.5)   | S (%) 0(0)     |
| Gentamicin           | S (%) 3(9.7)   | S (%) 8(15.7)  |
| Nalidixic acid       | S (%) 2(6.5)   | S (%) 0(0)     |
| Cephalothin          | S (%) 3(9.7)   | S (%) 0(0)     |
| Streptomycin         | S (%) 3(9.7)   | S (%) 24(47)   |
| Trimethoprim-sulfamethoxazole | S (%) 3(9.7) | S (%) 0(0)     |
| Ofloxacin            | S (%) 3(9.7)   | S (%) 0(0)     |
| Erythromycin         | S (%) 3(9.7)   | S (%) 0(0)     |
| Vancomycin           | S (%) 3(9.7)   | S (%) 0(0)     |

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; Stepansovic’s 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 (Stepansovic’s 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.

*1ISOKEN H. IGBINOSA
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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*ISOKEN H. IGBINOSA