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Research

Prevalence and antibiogram of *Salmonella* isolated from ready-to-eat fresh produce retailed in Umuahia, Nigeria

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Abstract

This study investigated the microbial load of fresh produce retailed in Umuahia, Nigeria, and assessed the prevalence and antibiotic resistance of *Salmonella*. The loads of bacteria and coliforms as well as presence of *Salmonella* in 42 fresh produce samples were determined by standard microbiological methods. Antimicrobial susceptibility profile of the *Salmonella* isolates was determined using disc diffusion assay, while the multidrug resistant isolates were assessed for tolerance to different concentrations of acetic acid (0.5-5%) and NaCl (1-5%). The total bacterial and coliform counts ranged from 7.42 to 8.59 and 4.75 to 6.53 $\log_{10}CFUg^{-1}$, respectively. *Salmonella* was detected in 30 (71.43%) samples. All 24 *Salmonella* isolates (100%) were resistant to amoxicillin, augmentin, cefuxoxime, cefuroxime and ceftazidime, while absolute susceptibility (100%) was only recorded for ofloxacin. Nine resistance patterns were demonstrated by the isolates, being resistant to at least 5/10 antibiotics and at most 8/10 antibiotics. All selected multidrug resistant isolates except *Salmonella* sp. cror1 survived in 5% NaCl, while no growth was observed for 7/8 isolates in 1.5% acetic acid. The high prevalence of *Salmonella* in retailed fresh produce and high frequency of multidrug resistance amongst the isolates suggest the need for increased awareness about hygienic practices and effective regulation of medically important antimicrobials.

Keywords: Antibiotics resistance, Fresh produce, Salmonella, Food Safety

Introduction

Fresh produce are farm-produced crops, including fruits and vegetables that are in the same state as where and when they were harvested. A significant proportion of fresh produce are not subjected to further processing before consumption. Epidemiologic and scientific evidences have established the protective roles of fresh produce in reducing the risk of several chronic diseases, including cancers, diabetes, obesity and coronary heart diseases, and promoting wellbeing (Van Duyn and Pivonka, 2000). They are important sources of bioactive and nutritional compounds, including minerals, phenolics, vitamins and dietary fiber (Schreinemachers *et al.,* 2018). This influenced National Dietary Guidelines and advisory bodies to favor increased consumption of green leafy cruciferous vegetables and wide varieties of fruits. For instance, the World Health Organization (WHO) recommends a minimum intake of 400 g per day of fresh produce (WHO, 2015).

Fresh produce harbour a wide range of spoilage and pathogenic microorganisms through various routes of contamination along the entire supply chain from farm-to-table. They are contaminated by direct contact with soil microflora, use of untreated wastewater and animal manure for irrigation and soil enrichment respectively, use of contaminated wash water and contact with vectors, unhygienic handlers and surfaces during processing, distribution and storage (Abakpa *et al.*, 2015; Mahajan *et al.,* 2017; Oyedele *et al.*, 2020). Furthermore, these contaminating microbes are able to proliferate in/on fresh produce to a magnitude that constitute public health and economic concerns. Contributory factors include the nutrient-rich internal tissues of fresh produce, warm and humid climate of the tropics and preference for minimal processing to conserve the inherent phytochemicals and nutrients in fresh produce (Mahajan *et al.,* 2017).

Among over 200 types of diseases spread by food, the occurrence of enteropathogens, including *Campylobacter* sp., *Escherichia coli*, *Salmonella* sp., *Shigella* sp. and *Listeria monocytogens* account for the greatest concerns with food-borne diseases outbreaks (WHO, 2015; Franz *et al.,* 2018). In Africa, *Escherichia coli* and *Salmonella* sp. have the highest prevalence in fresh produce (Paudyal *et al.,* 2017). *Salmonella* is a genus of rod-shaped, Gram negative and facultative anaerobic bacteria that contaminates diversity of foods from different environmental sources and a leading cause of foodborne diseases (Emond-Rheault *et al.,* 2017). Diseases due to consumption of *Salmonella*-contaminated foods are important causes of morbidity and mortality, and a significant contributor to socio-economic challenges (WHO, 2015).

The unregulated and indiscriminate use of antibiotics for growth promotion and diseases prevention in food-producing animals influences the acquisition and development of antibiotic resistance, especially among pathogenic microorganisms, including *Salmonella.* A grave public health and economic implication of multidrug resistant *Salmonella* is the increasing incidences of mortality, therapy failures and morbidity in patients and high health expenditure in humans and animals with severe infection (Abakpa *et al.,* 2015; WHO, 2017).

Sub-Saharan Africa has the challenges of poor agricultural practices for the cultivation of fresh produce, climatic conditions that favours microbial proliferation, limited resources to support cold supply chain, unregulated and indiscriminate use of antibiotics. The ready-to-eat nature of fresh produce implies it is imperative to generate baseline data on their level of contamination with *Salmonella*, particularly the multi-resistant strains. This is required to inform and guide the establishment, review and enforcement of food safety strategies targeted at cultivating, processing and retailing fresh produce and overall use of antibiotics. The aims of this study are to determine the occurrence and antibiotics resistance pattern of *Salmonella* in fresh produce retailed in Umuahia, Nigeria and evaluate their resistance of acidic- or osmotic stresses that could be used for decontamination purpose.

Materials and methods

Study area and sample locations

The study was carried out in Umuahia, Abia State, Nigeria which lies in the geographic coordinates of 5.5250 ˚N, 7.4922 ˚E. Umuahia is the administrative capital of Abia State in Eastern Nigeria with an average annual temperature of 26 ˚C and 10 months long annual rainfall, bringing the average precipitation to 2153 mm. Fresh produces are transported within 24-72 hours to the produce markets in Umuahia from the Northern part of Nigeria, where farmers rely on irrigation with available freshwater sources and animal manure application for cultivation. Four different spatially located fresh produce markets in Umuahia were selected for the purchase of fresh produce samples, including, Isigate, Ndioru, Orieugba and Ubani markets.

2.2 Sample collection

A total of 42 fresh produce samples were purchased from the four markets in Umuahia. The samples consisted of 12 samples each of carrot (*Daucus carota*), cucumber (*Cucumis sativus*) and lettuce (*Lactuca sativa*) and six samples of cabbage (*Brassica oleracea*). Two vendors were randomly selected in each of three different markets for each fresh produce and about 100 g of each sample was aseptically collected in two sampling rounds from the same vendors in June and July. The samples were placed in sterile plastic bags and immediately transported to the laboratory for bacteriological analyses within three hours of collection.

2.3 Bacteriological analyses

Samples were evaluated for total bacterial and coliform counts and screened for *Salmonella.* Twenty-five grams of each aseptically cut fresh produce sample was weighed into 225 mL of sterile 0.1% (w/v) bacteriological peptone and shaken vigorously for 3 minutes. Appropriate serial dilutions in the same diluent were spread-plated on nutrient and MacConkey agar for the cultivation and counting of total bacteria and coliforms respectively. The inoculated plates were incubated at 37 ˚C for 24 h (Biniam and Mogessie, 2010). For the detection of *Salmonella*, the remaining portion of the stock dilution was pre-enriched at 37 ˚C for 24 hours, then 1 mL of the enriched broth was transferred into 9 mL of sterile Selenite F Broth and selectively enriched at 37 °C for 24 h. A loopful each of the enriched medium was spread-plated on Salmonella-Shigella agar and incubated at 37 °C for 24 h (Abakpa *et al.*, 2015). Presumptive *Salmonella* colonies that appeared creamy or pinkish with central black spots were picked and preserved on nutrient agar slants at 4 ˚C for further confirmatory biochemical characterization, including, catalase, Gram staining, indole, citrate, H_2S production and triple sugar iron (TSI) tests. Standard methods were used to carry out the biochemical tests.

2.4 Antibiotics susceptibility profiling of *Salmonella* isolates

The antibiotic sensitivity profile of each *Salmonella* isolates was determined using the agar disc diffusion method of Kirby Bauer. Fresh culture of each isolate was suspended in 0.85% sterilized physiological saline solution and adjusted to 0.5 McFarland turbidity standard, equivalents to 1.5 X 10^8 CFU/mL. Mueller-Hinton agar plates were seeded with 0.2 mL of bacterial suspension by spread plate method with the aid of sterile cotton swab and left to dry for 15 minutes at room temperature. Commercially available antibiotics discs were aseptically placed on each seeded agar plates. The antibiotic discs were; Amoxicillin/ Clavulanic Acid (amc) (30µg), Amoxicillin (amx) (25µg), Ceftazidime (caz) (30µg), Cefuroxime (crx) (30µg), Gentamicin (gen) (10µg), Cefuxoxime (cxm) (5 µg), Ofloxacin (ofl) (5µg), Augmentin (aug) (30µg), Nitrofurantion (nit) (300 µg), Ciprofloxacin (cpr) (5 µg), Cefotaxime (ctx) (30 µg), Imipenem (ipm) (10µg), Perfloxacin (pef) (5µg) and Nalidixic acid (30µg). The plates were incubated for 24 hours at 37°C. The resultant diameter of visible zones of inhibition were measured in millimeters (mm) and classified as resistant (R), intermediate (I) or sensitive (S) in accordance to the guidelines

of the Clinical and Laboratory Standard Institute (2018).

2.5 Resistance to different concentrations of acetic acid and NaCl

Nutrient broths were spiked with 0.5 – 1.5 % (v/v) acetic acid or 1- 5 % (w/v) NaCl and inoculated with randomly selected isolates exhibiting multiple drug resistance to a cell concentration corresponding to 0.5 McFarland turbidity standard, equivalents to 1.5 X 10^8 CFU/mL. The broths were incubated at 37 ˚C for 24 h. Nutrient broth cultures of respective isolates without acetic acid and NaCl, incubated under the same conditions were used as control. Survival of isolates was evaluated by streaking out a loopful of the broth culture on nutrient agar plates and growth was observed after incubation at 37 ˚C for 24 h.

2.6 Statistical analysis

Data was collected in replicates and presented in mean ±standard deviation. Counts were expressed in logarithmic units of colonies per milliliters square ($\log_{10}$ CFU mL^{-1}).

Results

The total bacterial counts in 42 samples of fresh produce, including cabbage, carrot, cucumber and lettuce ranged from 7.42 to 8.59 $\log_{10}$ CFU g^{-1}. The coliform counts ranged from 4.75 to 6.53 $\log_{10}$ CFU g^{-1}. There were significant differences in the total bacteria and coliform counts for different types of fresh produce. The highest counts for total bacteria and coliforms were recorded for lettuce samples. Out of the 42 samples examined, 30 (71.43%) were contaminated with *Salmonella.* The highest number of *Salmonella* positive samples were carrot samples, with 11 (91. 67 %) of 12 samples being contaminated with *Salmonella* (Table 1).

Antibiotics susceptibility profiling of 24 *Salmonella* isolates recovered from the 42 samples of fresh produce revealed that all the isolates (100 %) were resistant to the two antibiotics in the penicillin class, including amoxicillin and augmentin and the three antibiotics in caphems class, including cefuxoxime, cefuroxime and ceftazidime (Table 2). Absolute sensitivity by all *Salmonella* isolates (100 %) was only recorded for ofloxacin, a quinolones and fluoroquinolones antibiotic class. In the case of two other antibiotics belonging to similar class as ofloxacin, 25 % and 37.5 % of the *Salmonella* isolates were sensitive to ciprofloxacin and perfloxacin respectively. The resistance profile of the isolates to gentamicin, an aminoglycoside was 8/24 (33.33 %) and the same value was recorded for nitrofurantoin, a nitrofuran (Table 2).

Antibiogram of Salmonella isolates to 10 antibiotics revealed nine resistance patterns. All 24 isolates exhibited multidrug resistance, each isolate being resistant to at least 5/10 antibiotics and at most 8/10 antibiotics, and with multidrug antibiotic resistance (MAR) index ranging from 0.5 to 0.8. The most frequent multidrug resistance pattern, against six antibiotics (AMC^R, AUG^R, CRX^R, CAZ^R, CXM^R, PEF^R) was demonstrated by seven *Salmonella* isolates from cabbage (1), carrot (1) and lettuce (5). The highest level of MAR (resistant to 8/10 antibiotics) was demonstrated by four *Salmonella* isolates which are distributed among cabbage (1), carrot (1) and cucumber (2) (Table 3).

The results in Table 4 showed the survival of the selected multidrug resistant isolates in different concentrations of acetic acid and NaCl. All the selected isolates survived in 0.5% acetic acid and 1 and 3 % NaCl. All test isolates except *Salmonella* sp. cbor1 survived in 1.0 % acetic acid, while all isolates except *Salmonella* sp. cror1 survived in 5 % NaCl. No growth was observed for 7/8 of the selected multidrug resistant *Salmonella* isolates in 1.5% acetic acid.

Table 1: Bacterial load and occurrence of *Salmonella* in fresh produce

Fresh produce	No of samples	Total bacterial count (log_{10} CFU g^{-1})	Coliform count (log_{10} CFU g^{-1})	No (%) *Salmonella* occurrence
Cabbage	6	8.14 ± 0.78	6.24 ± 0.09	4 (66.67)
Carrot	12	7.54 ± 0.55	4.82 ± 0.44	11 (91.67)
Cucumber	12	7.42 ± 0.62	4.75 ± 0.62	8 (66.67)
Lettuce	12	8.59 ± 0.46	6.53 ± 0.55	7 (58.33)
Total	42			30 (71.43)

Table 2: Antibiotics susceptibility profile of the *Salmonella* isolates from fresh produce

Antibiotics class	Antibiotics	Disc content (µg)	Fresh produce *n* = 24 (%)		
			Resistant	Intermediate	Sensitive
Penicillin	Amoxicillin	25	24 (100)	0 (0)	0 (0)
	Augmentin	30	24 (100)	0 (0)	0 (0)
Cephems	Cefuxoxime	5	24 (100)	0 (0)	0 (0)
	Cefuroxime	30	24 (100)	0 (0)	0 (0)
	Ceftazidime	30	24 (100)	0 (0)	0 (0)
Quinolones and fluoroquinolones	Ofloxacin	5	0 (0)	0 (0)	24 (100)
	Ciprofloxacin	5	1 (4.17)	17 (70.83)	6 (25)
	Perfloxacin	5	15 (62.5)	0 (0)	9 (37.5)
Aminoglycosides	Gentamicin	10	8 (33.33)	2 (8.33)	14 (58.33)
Nitrofurans	Nitrofurantion	300	8 (33.33)	11 (45.83)	5 (20.83)

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Table 3: Multidrug resistance profile of the *Salmonella* isolates from fresh produce

No. of antibiotics	Resistance phenotypes	MAR index	No. of antibiotics class	No of isolates				Total no. of isolates
				Cabbage	Carrot	Cucumber	Lettuce	
5	AMC, AUG, CRX, CAZ, CXM	0.5	2		3	1	1	5
6	AMC, AUG, CRX, CAZ, CXM, PEF	0.6	3	1	1		5	7
6	AMC, AUG, CRX, CAZ, CXM, GEN	0.6	3			1		1
6	AMC, AUG, CRX, CAZ, CXM, NIT	0.6	3	1	1			2
7	AMC, AUG, CRX, CAZ, CXM, CPR, PEF	0.7	3			1		1
7	AMC, AUG, CRX, CAZ, CXM, PEF, GEN	0.7	4	1		1		2
7	AMC, AUG, CRX, CAZ, CXM, PEF, NIT	0.7	4	1				1
7	AMC, AUG, CRX, CAZ, CXM, GEN, NIT	0.7	4	1				1
8	AMC, AUG, CRX, CAZ, CXM, PEF, GEN, NIT	0.8	5	1	1	2		4

Table 4: Survival of multidrug resistant *Salmonella* isolates from fresh produce in different concentrations of acetic acid and sodium chloride

Isolate	Source	Acetic acid		Sodium chloride			
		0.5%	1.0%	1.5%	1%	3%	5%
Salmonella sp. crnd1	Carrot	++	+	-	++	++	+
Salmonella sp. cror1	Carrot	++	++	-	++	+	-
Salmonella sp. cbor1	Cabbage	+	-	-	++	+	+
Salmonella sp. cbis2	Cabbage	++	+	-	+	+	+
Salmonella sp. ccnd1	Cucumber	+	+	+	+	+	+
Salmonella sp. ccnd2	Cucumber	++	+	-	++	++	+
Salmonella sp. ltub1	Lettuce	++	++	-	++	++	+
Salmonella sp. ltub3	Lettuce	++	+	-	++	+	+

++: Heavy growth, +: Moderate growth, -: No growth

Discussion

Microbial contamination of fresh produce occur along farm-to-table and the level of contamination depends on the agricultural practice and distribution and storage conditions (Mahajan *et al.,* 2017; Alegbeleye *et al.,* 2018). In this study, cabbage, carrot, cucumber and lettuce retailed in Umuahia markets were heavily contaminated. Apart from entry of microbial contaminants from soil, irrigation water and manures, the conditions during distribution and retailing of fresh produces in tropical countries favour further contamination and proliferation of microbes. The loads of total bacteria and coliforms reported in this study were also reported for lettuce and green pepper from Ethiopia (Biniam and Mogessie, 2010). Also, in consonance with our study, mixed fruits produced in Zambia had aerobic plate counts from 3.0 to 7.6 $\log_{10}$ CFU g^{-1} and coliform counts between 3.0 and 4.0 $\log_{10}$ CFU g^{-1} (Nguz *et al.*, 2005).

This study revealed the presence of *Salmonella* in 71.43% (30/42) of the fresh produce samples. Although no African data is available, *Salmonella* was reported to be responsible for more than 50% of fresh produce-borne outbreaks in North Africa (Emond-Rheault *et al.,* 2017). A study on green leafy lettuce in Thailand, with good agricultural practices and effective safety control programs reported 23.33% of samples to be positive for *Salmonella* (Chanseyha *et al.,* 2018). Analysis of publication in Africa by Paudyal *et al.* (2017) revealed average prevalence of *Salmonella* in 21.7 % of ready-to-eat (RTE) foods. High prevalence of *Salmonella* in fresh produce was earlier linked to the use of contaminated wastewater and untreated animal manure to respectively irrigate and enrich the soil used for cultivation (Abakpa *et al.,* 2015). The rate of *Salmonella* isolation in this study is higher than 13.9 % reported in a previous study, where the samples were picked directly from the farms. This suggests the unhygienic distribution conditions and the persistence of *Salmonella*, which is capable of surviving and proliferating in diverse environment, including conditions of transportation, storage and retailing (Akhtar *et al.,* 2010). Salmonella survives in complex ecological niches and harsh environments over a long period (Emond-Rheault *et al.,* 2017).

Multidrug resistance in *Salmonella* isolated from different sources have been widely reported, hence the first priority accorded this

genus for inclusion in surveillance of antimicrobial resistance in foodborne bacteria (WHO, 2017). This study reported resistance to amoxicillin, augmentin, cefuxoxime, cefuroxime and ceftazidime by all the *Salmonella* isolates. This is an indication of the indiscriminate use of antibiotics in clinical and veterinary practices (Okorie-Kanu *et al.*, 2016; Liu *et al.*, 2017). Resistance of *Salmonella* to various antibiotics was earlier reported by Biniam and Mogessie (2010). In agreement with this study, Abakpa *et al.* (2015) reported multidrug resistance in all isolates of *Salmonella* from vegetables and environmental samples in Northern Nigeria. Similarly, 100% resistance were reported for *Salmonella* strains of food origin in a review that summarizes data from countries in different regions of the world (Maka and Popowska, 2016). Chanseyha *et al.* (2018) also found *Salmonella* isolates from fresh leaf lettuce samples to be resistant to beta-lactams, tetracycline, trimethoprim and streptomycin antibiotics.

The majority of lettuce and green pepper samples considered in this study had high microbial load and, in some cases, even pathogens were isolated. As these are salad vegetables, they do not get any further heat treatment. The only control mechanisms are thorough washing and use of food grade chemicals to kill the microorganisms. Because of the high initial counts, reduction of number by washing or chemical treatment may still leave a good proportion of the microorganisms unaffected. Our study reported the sensitivity of *Salmonella* isolates to 1.5% acetic acid. Organic acids were previously mentioned to possess antibacterial efficacy against *Salmonella* spp (Nair *et al.*, 2018). Similar to our study, an organic acid, caprylate (15 mM), was found to be effective against *S. Typhimurium* and caused more than 4 log10 CFU/g reduction of *Salmonella* (Messens *et al.*, 2010).

Conclusion

This study reports the high prevalence of *Salmonella* in retailed fresh produce, suggesting poor agricultural practices in growing the crops, where untreated wastewater and animal manures are used as inputs. Besides, it indicates the absence of control and poor hygienic practices along fresh produce supply chain from the farm. There is need to increase the awareness of farmers and food distributors about good agricultural and hygienic practices respectively. This must be consolidated by adequately regulating their activities. The high frequency of multiple antimicrobial resistance in *Salmonella* isolates from this study made it imperative for local authorities to develop and adopt action plans and guidelines on the use of medically important antimicrobials in food-producing animals, plant production and humans. As ready-to-eat fresh produce are consumed without further microbiocidal processing, treatment with food grade antimicrobial preparations may contribute to reducing the load of microbial contaminants, particularly pathogens.

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