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Erwinia Rots and Presence of Pathogenic Bacteria in Symptomless Fruits and Vegetables

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Abstract: Spoilage and contamination of fruits and vegetables with pathogenic microorganisms constitute major agricultural and health concerns to both producers and consumers. The role played by *Erwinia* species in postharvest losses and the health associated risks in consuming fruits and vegetables without prior heat treatment were investigated. Fruits and vegetable samples collected from three municipal markets were cultured for the presence of *Erwinia* spp. and pathogenic bacteria. Pathogenicity study for *Erwinia* was carried out to demonstrate species specific predilection for certain crops. Antibiotic susceptibility of the isolated pathogenic bacteria was carried using the disc diffusion method. *Erwinia* spp. were isolated more from spoilt vegetables (80% -100%) than from spoilt fruits (65%-100%) and showed species preference for particular crop type. *Erwinia papayae* was isolated from *Carica papaya* (pawpaw) only, *E. amylovora* from *Malus domestica* (apple, 65%) and *Persea americana* (avocado pear, 100%), while *E. carotovora* was isolated from the vegetables; *Solanum tuberosum* (potato, 100%), *Solanum lycopersicum* (tomato, 90%), and *Alium cepa* (onion, 80%). Fruits and vegetables with no obvious symptoms of spoilage were contaminated with species of *Salmonella*, *Shigella*, *Escherichia*, *Klebsiella*, *Pseudomonas*, *Bacillus* and *Staphylococcus*. Multidrug resistance was found in some of the isolates especially to chloramphenicol, ciprofloxacin, erythromycin and tetracycline. Oxacillin resistance was as high as 29% in coagulase negative staphylococci. These findings underscore the importance of good hygiene and environmental sanitation in maintaining the keeping quality of fruits and vegetables postharvest.

Keywords: *Erwinia*, spoilage, fruits, vegetables, pathogens, postharvest

Introduction

Outbreaks of gastroenteritis and food poisoning have been reported in consumption of fruits or vegetables contaminated with toxigenic strains of *Bacillus cereus, Clostridium perfringens, Escherichia coli, Salmonella* spp. and *Staphylococcus aureus* [1-3]. *Erwinia* a genus in the Family Enterobacteriaceae has been implicated as major cause of postharvest rots in fruits and vegetables with serious economic consequences [4,5]. *Erwinia papayae* causes bacterial crown rot (BCR) in *Carica papaya*. Fire blight disease in apple and pear is caused by *E. amylovora* while *E. carotovora* is the cause of soft-rot diseases in carrots, potatoes, cucumbers, onions, tomatoes, lettuce, mustard and ornamental plants such as iris [6-8].

Fruits and vegetables are perishable crops with a short shelf-life [9]. Consequently, these cannot be stored in the raw form for prolong period in the fresh form without heavy chemical treatment [10,11]. The use of cold storage has limited application and is hindered by availability of constant electricity. Hygiene status of the storage facility; transportation conditions and handling, all contribute to the rapid deterioration of fruits and vegetables and entrance of pathogens. *Erwinia papayae* has been recovered from seed of infected papaya fruit and was shown to be viable in seed after an extraction and air drying process [12]. This indicates seed-borne transmission. Many peasant farmers cultivate seeds gleaned from fruits purchase from the market. This is an easy way of transmitting disease into a disease free orchard. Therefore, early detection can help prevent this occurrence. Moreover, outbreaks of gastroenteritis associated with consumption of seemingly healthy fruits or vegetables demand that frequent screening of pathogenic and spoilage organisms be carried out in order to reduce the health inherent risks by proffering methods of control [13,14]. In the present study we report on the pathogenesis of *Erwinia* species in selected fruits and vegetables and assess the health implications of consuming fruits or vegetables contaminated with pathogenic bacteria.

Materials and Methods

Culture Media

The media used in this study include: Nutrient agar, Nutrient broth, Peptone water, MacConkey agar, *Salmonella-Shigella* agar, Eosin Methylene Blue agar, Mannitol Salt agar, Mueller-Hinton agar, Simmon Citrate agar, Urea Base agar, MRVP broth, Sulphur Indole Motility (SIM) agar. These were products of BioMerieux and were prepared according to Manufacturer's instructions. Prepared media in sterile agar plates, agar slants in Bijou bottles, broth medium in sterile McCartney bottles and agar dips in screw capped test tubes were stored either at 6-8 ^{o}C or room temperature as appropriate. Sucrose Nutrient agar for the isolation of *Erwinia* spp. was prepared by adding 5 g of Sucrose to 95 ml of molten Nutrient agar and steam sterilized to give a 5% Sucrose Nutrient agar (SNA).

Antibiotics

Antibiotics used for the antibiotic susceptibility testing include; erythromycin (E, 15µg), penicillin (P, 10 IU), gentamicin (CN, 30µg), ciprofloxacin (CIP, 5µg), tetracycline (TE, 30µg), chloramphenicol (C, 30µg), oxacillin (Ox, 5µg), sulphamethoxazole/trimethoprim (SXT, 25 µg) and nalidixic acid (NAL, 30µg). The antibiotics were manufactured by

BioMerieux, France. Antibiotics discs were stored at 6-8 ^{o}C, and before use were allowed to attain room temperature (27-32 ^{o}C) before application onto the surface of inoculated Mueller-Hinton agar plates.

Table 1: Zone interpretive standards for the bacterial isolates

Antibiotics	S Interpretive Criteria (nearest whole mm)	R Interpretive Criteria (nearest whole mm)
Ciprofloxacin	≥ 21 (≥ 31-*Salmonella*)	≤15
Gentamicin	≥18 (≥15 – *Pseudomonas*)	≤12
Erythromycin	≥13	≤12
Tetracycline	≥15	≤11
Sulphamethoxazole/trimethoprim	≥16	≤10
Oxacillin	≥18	≤17
Penicillin	≥29	≤28
Chloramphenicol	≥18	≤12
Nalidixic acid	≥19	≤13

Key: S, susceptible; R, Resistant.
Clinical Laboratory Standards Institute, 2016 [15])

Collection of Samples

Two markets in Lagos (Jakande market in Ketu and Mile 12, along Ikorodu road) and Oja-Ota market Idiroko road in Ogun state) where fruits and vegetables are sold were selected for this study. Three fruits (*Malus domestica*, apple; *Persea americana*, avocado pear; *Carica papaya*, pawpaw) and three vegetables (*Solanum tuberosum*, potato; *Solanum lycopersicum*, tomato; *Allium cepa*, onion) were used for this study. Samples were collected twice weekly and over a period of 3 months. Infected and non-infected fruits and vegetables samples were collected in Ziploc bags rinsed in 2% sodium hypochlorite. The samples physical state was observed and spoilage type was recorded. Fruits with no visible sign of spoilage were classified as wholesome based on physical appearances. Healthy looking fruits but with breaks on the skin were grouped as injured fruits. Fruits or vegetable with outward healthy appearances but showed disease symptoms when pulped was recorded as inner spoilage. All spoilt samples and healthy samples within the different categories were cultured same day of sampling for the presence of *Erwinia* spp., Enterobacteriaceae, *Staphylococcus*, *Pseudomonas* and *Bacillus* spp. Three fruits (*Malus domestica*, apple; *Persea americana*, avocado pear; *Carica papaya*, pawpaw) and three vegetables (*Solanum tuberosum*, potato; *Solanum lycopersicum*, tomato; *Allium cepa*,

onion) were used for this study. Samples were collected twice weekly and over a period of 3 months. A total of 30 samples of each fruit type or vegetables were collected. Another set of healthy and disease free samples of apple, pawpaw, pear, potato, tomato, and onion were used for *Erwinia* pathogenicity study.

Processing of Samples for Culturing

The fruits and vegetables samples were rinsed with sterile water and dipped into sodium hypochlorite for surface disinfection. They were left at temperature of 16-21 ^{o}C (mean, 19 ^{o}C) in an air conditioned room to drip dry in a disinfected glass cabinet. A sterile scalpel blade was used to remove the skin (epidermal layer of the fruit and 1g of the underlining tissue was transferred into 9 ml of sterilized phosphate buffered saline in test tubes and further 10 fold serial dilutions were prepared. The fruits and vegetables were then pulped to observe the inner cavity for evidence of spoilage. Sterile scalpels were used in sampling the content of the cavity and dilutions prepared as described. Aliquots of 0.1 ml of the dilutions were inoculated onto the media for the isolation of either spoilage organisms or pathogenic bacteria. The plates were incubated at 37 ^{o}C for 18-48 h aerobically. Inoculated MacConkey agar plates, sucrose nutrient agar plates and nutrient agar plates for isolation of *Erwinia* species were incubated at two separate temperatures (28 ^{o}C and 37 ^{o}C). The streak and pour plate methods were employed. The pour plate technique was used mostly for microbial enumeration. Isolated organisms were identified by standard methods which include microscopy, cultural features and biochemical characterization.

Pathogenicity Study

The *Erwinia* isolates were assessed individually for pathogenicity. Bacterial suspension at a concentration of 1×10^{8} CFU per ml was prepared and inoculated into healthy fruits as described below. The surfaces of disease free fruits or vegetables were disinfected by swabbing with sterilized cotton swab soaked with sodium hypochlorite. The test samples were divided into two categories; wounded and unwounded samples. For wounded samples, wounding was artificially created by incision with sterile scalpel. Wounding was done to simulate the effect of breaks on fruit skin on the rate and degree of spoilage. Rectangular sterile plastic discs were soaked in the bacterial suspensions, dried and used as the source of inoculums. These were placed on the wounded and unwounded sections of the samples and stored in an air conditioned room with mean temperature of 19 ^{o}C for one set and for another at room temperature with mean of 32 ^{o}C. The progression of the disease symptoms was observed and recorded from day three.

Antibiotics Susceptibility Test

Pure culture (18-24 h) of the sample on a non-selective medium was prepared. The turbidity was adjusted to 0.5 McFarland Turbidity Standards in phosphate buffered saline (pH 7.2). The surface of Mueller-Hinton agar plates were inoculated with albumin coated swabs soaked with the inoculums. Antibiotic discs were placed on the surface of the seeded Mueller-Hinton agar and incubated aerobically at 37 ^{o}C for 18 h. Zones of inhibition were measured and interpreted against the CLSI 2016 standards [15] (Table 1).

Results

The microbial load of adhering surface flora of fruits and vegetables from the three markets after disinfection as described in this study is given in Table 2. The values indicate that samples from Jakande-Ketu market had the highest microbial load compared with those from the other markets. Tomato had the highest microbial count 4.0 x 10^8 CFU/ml while the least count was from onion 1.8 x 10^3 CFU/ml.

Tables 3 and 4 show percentage distribution of pathogenic bacteria in the three categories of fruits and vegetables studied. Generally, fruits with inner spoilage had the highest incidence of pathogenic bacteria, followed by fruits with breaks on their skin. However, some fruits without obvious spoilage symptoms and no noticeable breaks on the skin grew pathogenic bacteria especially *Bacillus* spp. Genera in Enterobacteriaceae were predominant; however, *Shigella* was the least encountered in the fruits and vegetables.

Table 5 gives the resistance of the pathogens to some antibiotics. *E. coli* gave moderate 30.7% and high level 61.5% resistances to erythromycin and tetracycline respectively. With the exception of ciprofloxacin for which zero resistance was recorded, *P. aeruginosa* showed varied resistance to other antibiotics (8.7% for nalidixic acid and tetracycline) and 26.1% to chloramphenicol. *Klebsiella pneumoniae, Salmonella* and *Shigella* also recorded high level resistances against erythromycin and tetracycline. Ciprofloxacin resistance was seen in 83.3% *Salmonella* isolates and 66.7% *Shigella* isolates. Zero resistance to gentamicin and ciprofloxacin was recorded against the staphylococci. For other antibiotics, the coagulase negative staphylococci recorded higher resistances compared with *S. aureus*. Oxacillin resistance of 29% was seen only in coagulase negative staphylococci. *Erwinia amylovora* was the only species isolated from apple (65%) and pear (100%), while *E. carotovora* was isolated from potato (100%), onion (80%) and tomato (90%). *Erwinia papayae* was isolated from 75% of pawpaw samples (Table not shown). The disease symptoms associated with the various *Erwinia* species are depicted in Figure 1. The pathogenic capacity of *Erwinia* spp. for preferred hosts is indicated in Figures 2 and 3 and plates 1-4. Disease progression and severity was more pronounced at 32 ^oC except for potato where disease severity and progression was enhanced at 19 ^oC.

Table 2: Microbial load of fruits and vegetables from different markets

Samples	*Market (CFU/ml) A	B	C
Apple	3.5 x 10^7	2.7 x 10^6	6.8 x 10^6
Pear	9.3 x 10^5	9.7 x 10^4	2.8 x10^4
Pawpaw	1.3 x 10^3	1.0 x 10^2	4.6 x 10^2
Potato	1.3 x 10^6	1.7 x 10^5	3.0 x 10^5
Tomato	4.0 x 10^8	2.6 x 10^8	2.0 x 10^8
Onions	1.8 x10^3	3.8 x 10^2	3.0 x 10^2

Keys: A, Jakande – Ketu, Lagos state; B, Mile 12, Lagos state; C, Ota, Ogun state; *Mean count

Table 3: Distribution of pathogenic bacteria in spoilt and wholesome fruits

Isolate	Percentage distribution of bacteria in fruits								
	Apple			Pear			Pawpaw		
	IF (40)	IS (30)	WS (30)	IF (45)	IS (25)	WS (30)	IF (40)	IS (30)	WS (30)
Escherichia coli	27.5	26.7	10	26.7	36	13.3	32.5	26.7	3.3
Staphylococcus spp.	12.5	23.3	23.3	28.9	44	20	30	33.3	16.7
Staphylococcus aureus	70	56.7	16.7	48.9	60	10	30	30	6.7
Pseudomonas aeruginosa	37.5	50	10	22.2	60	6.7	32.5	23.3	3.3
*Klebsiella pneumonia*e	22.5	6.7	10	20	32	16.7	22.5	26.7	10
Enterobacter spp.	25	33.3	0	26.7	52	6.7	27.5	33.3	6.7
Salmonella spp.	17.5	26.7	16.7	6.7	36	23.3	15	30	0
Shigella spp.	7.5	0	0	4.4	0	3.3	5	0	0
Bacillus subtilis	25	5	6.7	0	15.6	44	13.3	25	13.3
Bacillus cereus	37.5	30	3.3	0	36	3.3	0	40	3.3

Key: IF, injured fruits; IS, fruits with inner spoilage; WS, wholesome fruits, *values in parentheses are numbers of samples studied

Table 4: Distribution of pathogenic bacteria in spoilt and wholesome vegetables

Isolate	Percentage distribution in vegetables								
	Potato			Tomato			Onion		
	IV (40)	IS (30)	WV (30)	IV (45)	IS (25)	WV (30)	IV (40)	IS (30)	WV (30)
E. coli	17.5	26.7	6.7	28.9	36	6.7	52	6.7	3.3
Staphylococcus spp.	20	23.3	6.7	26.7	44	26.7	0	33.3	10
S. aureus	22.5	56.7	16.7	48.9	60	23.3	12.5	30	10
P. aeruginosa	25	50	20	20	60	16.7	12.5	23.3	13.3
K. pneumoniae	22.5	6.7	0	22.2	32	10	22.5	26.7	6.7
Enterobacter spp.	7.5	33.3	6.7	26.7	52	23.3	10	33.3	3.3
Salmonella spp.	52	6.7	16.7	6.7	36	10	0	30	6.7
Shigella spp.	0	0	0	4.4	0	6.7	5	0	0
B. subtilis	25	56.7	0	15.6	44	10	25	13.3	20
B. cereus	7.5	30	0	0	36	0	0	40	13.3

Keys: IV, injured vegetables; IS, vegetables with inner spoilage; WV, wholesome vegetables; *values in parentheses are numbers of samples studied

Table 5: Resistance profile of the isolates to some antibiotics

Isolates (number)	Number (%) Resistance						
	C	CIP	CN	SXT	E	TE	NAL
E. coli (13)	0(0)	0(0)	0(0)	0(0)	8(61.5)	4(30.7)	0(0)
P. aeruginosa (23)	6(26.1)	0(0)	4(17.4)	5(21.7)	3(13.0)	2(8.7)	2(8.7)
K. pneumoniae (16)	2(12.5)	0(0)	0(0)	0(0)	16(100)	16(100)	0(0)
Enterobacter spp. (14)	0(0)	0(0)	0(0)	0(0)	7(50)	3(21.4)	0(0)
Salmonella spp. (18)	2(11.1)	15(83.3)	0(0)	0(0)	18(100)	17(94.4)	2(11.1)
Shigella (3)	0(0)	2(66.7)	1(33.3)	0(0)	3(100)	2(66.7)	1(33.3)
	CN	CIP	SXT	E	TE	OX	P
Staphylococcus spp. (31)	0(0)	0(0)	15(48.4)	3(9.6)	13(41.9)	9(29.0)	31(100)
S. aureus (25)	0(0)	0(0)	25(100)	0(0)	25(100)	0(0)	25(100)

Key: C, chloramphenicol; CIP, ciprofloxacin; N, gentamicn; E, erythromycin; TE, tetracycline; Nal, nalidixic acid; OX, oxacillin; P, penicillin; SXT, sulfamethoxazole-trimethoprim

Spoilt fruit/vegetable sample	Description	Organism isolated
	Ripe Papaya with reddish colouration and sunken lesion	*Erwinia papayae*
	Unripe papaya with sooty rots and sunken lesion	*Erwinia papayae*
	Ripe Papaya with soft rot symptoms	*Erwinia papayae*
	Affected pear fruit with water soaked appearance, wilt, shrivel and scorched	*Erwinia amylovora*
	Affected pear fruits with water soaked appearance and turn brown to black as the infection advanced	*Erwinia amylovora*
	Affected Apple fruits with sunken lesion	*Erwinia amylovora*
	Potato with soft rot and white fluffy rot symptoms	*Erwinia carotovora*
	Infected potato with inner soft rot	*Erwinia carotovora*

	Tomato with soft rot *Erwinia carotovora* symptoms
	Tomato with soft rot and *Erwinia carotovora* water soaked lesion
	Onion with inner soft rot *Erwinia carotovora* symptoms
	Onion with soft rot *Erwinia carotovora* symptoms

Figure 1: Fruits and vegetables with spoilage description and organisms isolated

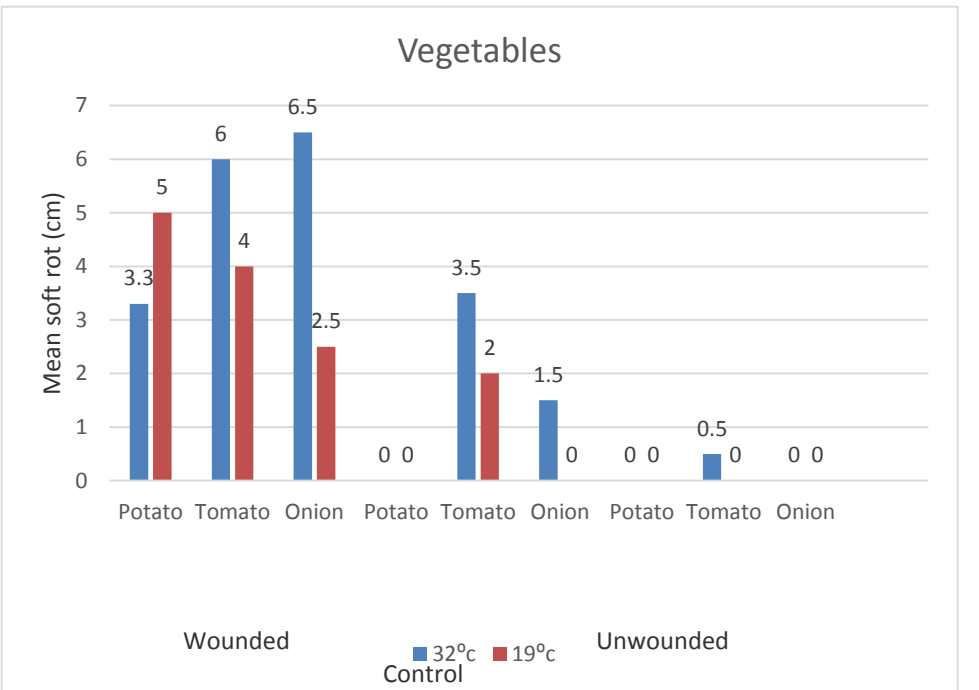

Figure 2: Diameter of diseased section of vegetables following inoculation with isolated *Erwinia* species

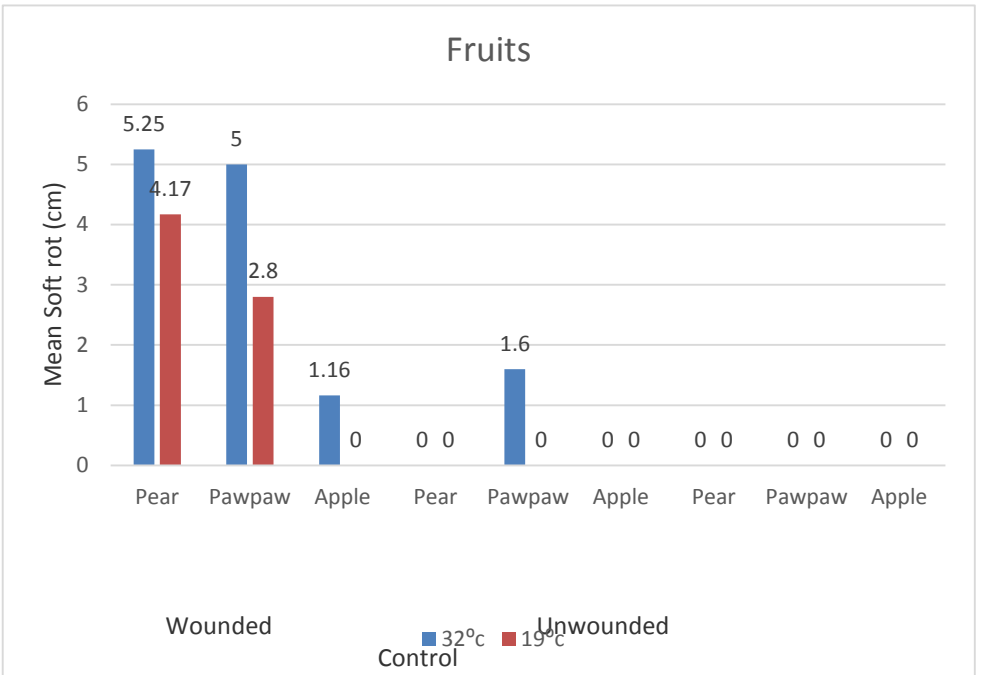

Figure 3: Diameter of diseased section of fruits following inoculation with isolated *Erwinia* species

Plate 1: Pathogenic effect of *E. amylovora* on healthy pear without artificial wounding at 32 ^{o}C three days after inoculation

Plate 2: Un-inoculated healthy pear fruit stored at 32 ^{o}C for three days

Plate 3: Disease symptoms (scorch fire appearance) caused by *E. carotovora* day five on wounded healthy potato stored at 19 ^{o}C

Plate 4: Enhanced pathogenicity of *E carotovora* on wounded healthy potato on day five after inoculation and stored 32 ^{o}C.

Discussion

Fruits and vegetables are consumed for their rich contents of energy, vitamins and minerals. These benefits can be diminished as a result of contamination with spoilage or pathogenic microorganisms. In this study, wholesome fruits and vegetables were analyzed for presence of microorganisms. The microorganisms isolated are direct reflections of the deplorable state of sanitation and hygiene of the environment and the vendors of these farm produces as evident in the high microbial counts of samples from the markets. Other contributing factors which could not be measured in this study but well documented in literature as predisposing to food contamination with microbes are processing water, harvesting techniques, transportation and storage conditions [16,17]. Since these food items are eaten in the fresh form or receive minimal heat treatment, the presence of pathogens constitutes health hazard. Postharvest rots remain a limiting factor to both the nutritional benefit and industrial use of fruits and vegetables. In the present study, *Erwinia* was observed to play a major role in the postharvest rots of fruits and vegetables; thus highlighting the agricultural significance of this bacterium.

This study demonstrated that *Erwinia* species are major postharvest spoilage organisms in fruits and vegetables. Therefore, methods of controlling their survival and proliferation in fruits and vegetables postharvest should be implemented. Available methods of control which have yielded some measurable degree of success include phytosanitation [18,19], chemical control with copper hydroxide [20], disinfection either with potassium-

manganous oxide (5%) and a quaternary ammonium compound at concentration of 10%, or sodium hypochlorite [21]. However, the health implication of the use of these chemicals in control of *Erwinia* rots in fruits and vegetables should be considered. *Erwinia carotovora* showed great predilection for potato, tomato and onion, indicating its wide host range as opposed to *E. papayae* and *E. amylovora* which have narrow host range [22]. This was further corroborated in the pathogenicity study that showed *E. carotovora* aggressiveness for both potato and pawpaw.

All the bacteria isolated in this study have previously been isolated from fruits and vegetables in other studies, both in Nigeria and elsewhere [2, 23-27]. However, the present study in addition, showed that wholesome looking fruits contained microorganisms that are of public health concern such as *Salmonella* and *Shigella*. This therefore calls for caution in the consumption of raw fruits and vegetables. *Pseudomonas* spp. and *Bacillus* spp. are part of the natural flora and are among the most common vegetable spoilage bacteria [28], though some *Bacillus* species (*B. cereus*) are capable of causing foodborne illness. The medical importance of most of the organisms isolated in this study requires that good hygiene practice and environmental sanitation be observed to safeguard the health of the consumers of farm produce.

The results for the antibiotic susceptibility test indicated that fruits and vegetables may be media for the spread of antibiotic resistant bacteria. A major finding is the resistance of a large percentage of *Salmonella* and *Shigella* isolates to ciprofloxacin a first line drug for the treatment of infections associated with these pathogens. The variation in the susceptibility of these organisms towards antibiotics may be connected to their previous exposure to the antibiotics [29]. These resistant strain especially the multi-drug resistance, are mostly implicated in epidemics [30].

Conclusion

This study has established *Erwinia* as a major postharvest spoilage organism of fruits and vegetables resulting in reduction of their market value. Secondly, the resistant pattern of the pathogens to antibiotics confirmed that drug resistant organisms can be spread through food sources. Therefore, fruit and vegetable processors should be educated on the adverse effect of using untreated or polluted water for processing as these could serve as sources of contamination. Also, is the need to develop new, safe and acceptable postharvest disinfestation methods; but most importantly, improving on hygiene and sanitation should be emphasized at all time. These will help farmers, marketers and consumers take the necessary precautions in preventing contamination of fruits and vegetables, thus reducing the risk of spread of infections and possible epidemics.

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