Prevalence of Gram-negative bacteria in Fresh Vegetables on retail in the Open Market in Owo Township, Ondo State, Nigeria

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Abstract

Fresh vegetables serve as major sources of micronutrients yielding critical vitamins, phytochemicals and essential minerals that improve wellness and supportive in staving-off non-communicable diseases, NCDs. Vegetables are generally cultivated in soils that also contain resident microorganisms that may contaminate them directly or shed from human and animal activities. The current study intended to evaluate fresh raw vegetables on retail in the open market in Owo Township, Ondo State, Nigeria for the presence of the Gram-negative bacterial flora. Twelve samples of six different types of vegetables collected and examined, applying bacteriological techniques for microbial count and assortments of Gram-negative bacteria. The mean bacterial load ranged from 3.66-111.0 x 10^6 CFU/g and seven genera of Gram-negative bacteria recovered. *Klebsiella* spp, 22 representing 25% of the isolates was the most dominant microorganism. *Escherichia coli* 20(22.7%) was the next in frequency from which an isolate serotyped 0157:H7 strain. Other isolates were *Enterobacter* spp 17(19.3%) *Proteus* spp 15(17.0%), *Pseudomonas aeruginosa* 10(11.4%) and two, 2(2.3%) isolates each of *Serratia marcescens* and *Salmonella enterica*. The isolates were most susceptible to ciprofloxacin and levofloxacin, and least to amoxicillin-clavulanate. All the isolates were resistant to amoxicillin and tetracycline. Extended-spectrum beta-lactamases were detectable in five of the seven genera (2.3-4.5%) excluding *Serratia marcescens* and *Samlonella enterica* isolates. This study exposed evidence of vegetables pollution with microorganisms associated with foodborne outbreaks of illnesses, compelling an urgency for education in handling practices compatible with the safety of fresh vegetables and related farm produce.

Introduction

Vegetables represent a vital source of vitamins, minerals including phytochemicals necessary for the modulation of anti-oxidative activity and dietary fiber when consumed in adequate quantities and regularity. They in addition, help to reduce the incidence of non-communicable diseases, NCDs as obesity, cardiovascular diseases, CVDs and neoplasms [1-7]. Leafy vegetables consist of a wide variety of nutrients that aid digestion and prevent oxidative stress [8-10]. The World Health Organization, in recognition of the pivotal role vegetables play in humans' wellbeing recommends 400 grams in five daily servings of vegetables and fruits and early introduction during childhood [11,12]. Leafy vegetables like many other plants possess adhering particles, APs that aid the contamination with particulate matter that can include bacteria and parasites amongst others, even after washing [13,14]. Microorganisms are constantly present in most human environments and abundant in the sites for the production of vegetables where the get access and become potential vehicles for the transmission of food-borne diseases [15-17]. There are reports that microorganisms can be shed into vegetables and fruits during processing and handling stages [18]. Vegetables laden with microorganisms especially *Salmonella* species, (*Salmonella* spp) *Shigella* species (*Shigella* spp) and enterotoxigenic strains of *Escherichia coli* (*E. coli*) pose remarkable health hazards to consumers [19,20]. Consumption of raw or partially cooked vegetables yields greater nutritional benefits, however, when this happens under unsanitary conditions, food-related illness may result [21]. The presence of antimicrobial resistant, AMR Gram-negative bacteria have been reported in vegetables to have grave repercussions for the health of consumers in terms of elevated costs of treatment, reduced chances of treatment success and the consequential increase in mortality rates [22-25]. Infections emanating from microorganisms contaminating food commonly manifest as diarrheagenic illnesses with greater severity and often top-ranking cause of higher mortality rates in children under 5 years 26-28]. This study aimed to examine fresh raw vegetables on retail in the open market in Owo Township for the presence of Gram-

Materials and Methods

Study Design:

A cross-sectional study to determine the prevalence Gram-negative bacteria flora of fresh raw vegetables on retail in the open market in Owo Township, Ondo State, Nigeria in June 2024.

Collection of Samples

Ninety-six (96) samples of six common varieties of vegetables in retail bunches in the open market in Owo Township were randomly procured from retailers; namely fluted pumpkin (*Telfairia occidentalis*), water leaf (*Talinum triangulare*), bitter leaf (*Vernonia amygdalina*), African spinach (*Amaranthus cruentus*), spring onions (*Allum fistulosum*) and cabbage (*Brassica oleracae*) into sterile autoclavable polythene bags and stored in a cold chamber. The samples were thereafter, transported to the laboratory for examination within 2 h of collection.

Examination of Samples

Ten gram (10 g) of each vegetable sample was weighed aseptically into 90 mL of Buffered Peptone Water, BPW (Oxoid CM 1049), and placed on mechanical shaker for 5 min from which 0.1 mL each was inoculated onto MacConkey agar, MCC (Oxoid CM 0007) and Salmonella-Shigella agar, SSA (Oxoid CM 0099). Further ten-fold serial dilutions from the

stock (10^2) to yield 10^3. 10^4, 10^5…10^{10} was done and 0.1 mL of each dilution was inoculated onto well-dried plates of Trypicase soya agar, TSA (Oxoid PO 0163A) in replicates. One set of plates incubated at 37^oC and the other at room temperature, RT. The cultures were examined after 24 h. Cultures with insufficient growth were re-incubated for examination at 48 h and 72 h. The colony forming units, CFU appearing on the TSA plates were enumerated and mean count determined. Colonies appearing on MCC and SSA plates picked for characterization and identification tests following the scheme described by Cowan [29]. Isolates susceptibility to tetracycline (TE, 10 µg), amoxicillin (A, 10 µg), amoxicillin-clavulanate (AMC, 30 µg), gentamycin (CN, 10 µg), ciprofloxacin (CIP, 10 µg), levofloxacin (LVX, 10 µg), ceftazidime (CAZ, 30 µg), cefotaxime (CAZ, 30 µg) and azithromycin (AZM, 15 µg) determined using the Kirby-Bauer disc diffusion method applying the guidelines of the Clinical Laboratory Standards Institute [30].

Results

The microbial load of sampled vegetables ranges from 3.7-111.0 x 10^6 CFU/g (Table 1). The highest mean count occurred in samples of fluted pumpkin, 111.0 x 10^6 CFU/g, followed by waterleaf with a mean count of 79.5 x 10^6 cfu/g and the least count of 3.60 x 10^6 CFU/g recorded from cabbage. The distribution of Gram-negative bacteria isolated presented in Table 2 showed that *Klebsiella* species (*Klebsiella* spp), 22(25%) of the total 88 isolates was the most predominant Gram-negative bacterium, of these 22 isolates, *Klebsiella pneumoniae* were 11(12.5%), *Klebsiella aerogenes* 9(10.2%) and *Klebsiella oxytoca* 2(2.3%). *E. coli* 20(22.7%) was the next frequently encountered microorganism of which one isolate serotyped *E. coli* 0157:H7. Other Gram-negative bacteria isolated were *Enterobacter* species (*Enterobacter* spp.) 17(19.3%) consisting of *Enerobacter cloacae* 11(12.5%) and *Enterobacter aerogenes* 6(6.8%); *Proteus* species (*Proteus* spp.) 15(17.0%) were made up of *Proteus mirabilis* 12(13.6%) and *Proteus vulgaris* 5(5,7%); *Pseudomonas aeruginosa* 10(11.4%). The least frequently isolated microorganisms were *Serratia marcescens* and *Salmonella enterica* with 2(2.3%) isolates each.

Antimicrobial susceptibility of the isolates displayed in Table 3 indicates that no isolates was susceptible to tetracycline. *E coli*, *Klebsiella* spp. and *Proteus* spp were most susceptible to gentamycin, ciprofloxacin and levofloxacin (54.5-95.0%) and least to amoxicillin and amoxicillin-clavulanate. *Pseudomonas aeruginosa* was most susceptible to gentamycin. *Serratia marcescens* susceptibility was highest with levofloxacin, ceftazidime and cefotaxime while *Salmonella enterica* was resistant to only amoxicillin. All isolates were resistant to tetracycline and amoxicillin.

Extended-spectrum beta-lactamases, ESBLs detection was highest in isolates of *E. coli* at 4.5%, *Klebsiella* spp (3.4%), *Enterobacter* spp 3.4%, *Proteus* spp 2.3%; and *Serratia marcescens* 2.3%. and *Salmonella. enterica* isolates tested negative for ESBLs.

Table 1: Microbial load of fresh vegetables

Vegetables	Mean CFU/g x 10^6)
Fluted pumpkin	111.0
Water leaf	79.3
Bitter leaf	72.1
African spinach	65.5
Spring onions	34.0
Cabbage	13.6

Tables 2: Distribution of isolates in different types of vegetables

Isolate	Frequency of isolates					
	Fluted pumpkin	Waterleaf	Bitter leaf	African spinach	Spring onions	Cabbage
E. coli (n=20)	3	4	1	3	5	4
Klebsiella spp (n=22)	4	5	3	6	2	2
Enterobacter spp (n=17)	2	5	3	1	3	3
Proteus spp (n=15)	3	2	3	2	1	4
Pseudomonas spp (n=10)	2	0	2	1	3	2
Serratia spp (n=2)	1	0	0	0	0	1
Salmonella spp (n=2)	0	0	0	0	1	0

Table 3: Susceptibility of isolates to antimicrobial agents

Isolate	TE	A	AMC	CN	CIP	LVX	CAZ	CTX	AZM
E. coli	0.0	15.0	25.0	70	90.0	95.0	60.0	65.0	55.0
Klebsiella spp	0.0	0.0	13.6	54.5	77.3	90.9	45.5	50.0	45.5
Enterobacter spp	0.0	0.0	5.6	66.1	83.3	88.9	50.0	55.6	50.0
Proteus spp	0.0	0,0	12.5	62.5	77.8	83.3	44.4	43.8	37.5
Pseudomonas spp	0.0	0.0	80.0	50.0	50.0	40.0	50.0	50.0	10.0
Serratia spp	0.0	0.0	0.0	50.0	50.0	100	100	100	50.0
Salmonella enterica	0.0	0.0	100	100	100	100	100	100	100

Discussion

Examination of fresh vegetables procured from the open market in Owo Township revealed bacterial loads of 3.0-111.0 x 10^6 CFU/g. These values are in gross excess of the upper limit of 10^2 CFU/g [31]. Prior studies in Nigeria and elsewhere reported similarly high values [19,23,24]. Vegetables by nature like other leafy plants possess adhering surfaces that make colonization and pollution easier, which necessitates greater adherence and compliance with strict standardized operational procedures, SOPs from the field to harvesting, packaging, transportation, and distribution and retail stages to minimize high bacteria counts in vegetables. Three genera of the isolated microorganisms - *Klebsiella* spp *Pseudomonas aeruginosa* and *Enterobacter* spp belong to the group of bacteria that pose major challenges in the treatment of their infections and as well as belonging to the ESKAPE bacteria group (*Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp) recognized worldwide for high resistance to antimicrobial agents. The absence of effective treatment options in several of the infections with these microorganisms makes it a major source of health peril [32,33]. This has in consequence made the call for newer and more effective antimicrobial agents more urgent and strident for mitigation in such infections as they are often accompanied with greater treatment budgets and elevated mortality rates [34-37]. Other key isolates - *E. coli* and *Salmonella enterica* are important in food-related outbreaks of illnesses [38-41]. While *Proteus* spp. and *Serratia marcescens* have the reputation for contaminating vegetables and fruits, in addition to possessing the tendency for resistance to many antimicrobial agents [17,42-48]. *Klebsiella* spp was the most predominant 22(25.0%) of 88 isolates with a proportionately higher numbers of *Klebsiella pnuemoniae* (12.5%) and *Klebsiella aerogenes* (10.2%) than *Klebsiella oxytoca* (2.3%) regularly contaminate vegetables. Contamination of fresh vegetables by *Klebsiella pneumoniae* have been reported as common due to the possession of enhanced ability by this microorganism to colonize bodies of natural water, sewage, organic effluents, vegetation, in addition to being a commensal in the gut of humans and animals [49,50]. Each of these isolates belong to important group of Enterobacteriaceae involved in causing a multiplicity of human infections, ranging from urinary tract infections, UTIs, pulmonary infections to hospital acquired infections, HAIs, chiefly in patients with preexisting disorders [51,52]. *E. coli* (22.7%) was the next most frequently encountered microorganism. The classical habitat of *E.*

coli is the gut of humans, animals and birds. This microorganism is a major etiological agent in uncomplicated UTIs and numerous other human infections. The detection of *E. coli* in food is an index of fecal pollution [53-56] and the probable presence and transmission of other dangerous bacteria, parasites and viruses from such vegetables. The detection of *E. coli* O157:H7 or Enteropathogenic, EPEC strain from a sample of cabbage is an indication of significant threat to the health of consumers of such vegetables. *E. coli* O157:H7 is an agent of diarrheagenic illness that induces abdominal cramps, watery and bloody stools and linked to several foodborne outbreaks of illnesses in many countries [31,38.41]. The infection in children results in more intensive disease process with high mortality rates under the age of 5 years [57,58], and especially in Africa, Asia and Latin America where hygiene levels are lower and health development systems are poorer or inadequate [26]. The entry route of *E. coli* O157:H7 into the food chain is traceable to fecal contamination from the cattle reservoir [39,59-61]. Studies have shown that pathogenic microorganisms including *E. coli* O157:H7 can contaminate vegetables at several stages before getting to the dining table [40,62,63]. This is more likely to happen in the study community due to unsanitary handling practices and the absence of ready source of potable water to vegetable farmers and handlers. Isolates of *Enterobacter* spp represented 19.3% consisting of *Enterobacter cloacae* and *Enterobacter aerogenes* as a major contaminant of these vegetables. *Enterobacter* spp colonize fresh water, soil, sewage and organic effluents and its presence in fresh vegetables is therefore not surprising. As a member of the ESKAPE group of bacteria known for resistance to a wide variety of antimicrobial agents and associated with serious hospital-related infections galvanized the World Health Organization into listing the microorganism into the group of priority bacteria requiring the sourcing for new treatment options are careful control strategies [56-65]. The presence of *Enterobacter* spp in vegetables is thus a worrisome finding as it denotes a source of health hazard to consumers [25,58]. *Proteus mirabilis* and *Proteus vulgaris* as well as other Enterobacteria have been observed as prevalent in fresh raw vegetables as they share common habitats [66], posing similar health challenges as the ESKAPE group of bacteria [32,36.42]. *Pseudomonas aeruginosa* is an underrated pathogen causing food spoilage even in low temperatures (psychrotrophic) [67]. The microorganism is environmentally common, often contaminating vegetables with most strains being MDR, can become channels for the spread of MDR infectious bacteria [68]. This further emphasizes the all-important task for the protection of food sources from pollution by dangerous microorganisms [16,32]. The recovery of *Serratia marcescens*, being a common microorganism found in the soil and in decaying vegetation, with the absence of appropriate standard safety measures, ultimately leads to easy contamination of vegetables and fresh farm produce [44-46,69]. The microorganism is frequently associated with opportunistic infections [48] as well as contributing to food deterioration and spoilage [47]. Many isolates of *Serratia marcescens* are MDR strains that makes their presence in food a source of concern. *Salmonella enterica* (2.3%) ware recovered from cabbage and spring onions, fascinatingly, one the least isolates encountered and a classical initiator of food-related outbreaks of diseases. *Salmonella enterica* can gain access to fresh produce like vegetables in the farm from the application of organic manure, polluted irrigation water, animal feces and introduction during processing and handling stages [70-72]. The association of several Gram-negative bacteria including *Salmonella enterica* did not come as a shock, but rather, it reflects the abysmally low level of hygiene from the farm to the retail point. Enteric illnesses are common during the rainy seasons and poorly reported too, in most parts of Nigeria, thus making it difficult to have the precise data. This study brings to fore the wide gap in the knowledge for sanitary processing and handling of fresh vegetables and the essence for urgent education before vegetables get to the dining table. The susceptibility of the four most common isolates ranged from 54.5-95.0% for gentamycin, ciprofloxaxin and levofloxacin and least to amoxicillin and amoxicillin-clavulanate. These microorganisms belong to the class

of microorganisms in WHO priority list due resistance to multiple groups of antimicrobial agents and antecedent elevated risk of treatment failure and associated higher fatality rates [73-75]. The higher rates of susceptibility comparatively to those obtainable in clinical samples reflect their origin as environmental microorganisms with limited exposure to antimicrobial agents arising from several strata of hurdles between humans, animals and the natural environment [76]. *Pseudomonas aeruginosa* was most susceptible to gentamycin (80%). *Serratia marcescens* had higher susceptibility to levofloxacin, ceftazidime and cefotaxime at 100%. The susceptibility of these isolates were also higher than those reported from clinical specimens, and in spite this, they are still largely regarded as a source of peril to public health due the MDR signature of members of these genera [21,25,77-81]. Expectedly, no isolate was susceptible to tetracycline as earlier studies have shown that environmental acquired ESKAPE and other Enterobacteriaceae are regularly resistant to the agents [82,83]. The detection of ESBLs in the isolates ranged from 2.3% to 4.5% in *E. coli* isolates. This may indicate that some of the isolates may have originated from human sources [84], thus further amplifying the anxiety on the probable treatment failure in the use of antimicrobial agents. Prior observations indicate that ESBLs producing Enterobacteriaceae also exists in the environment and can contaminate vegetables [24,85]. The detection of ESBLs among *Klebsiella pneumoniae* and *Klebsiella aerogeges* isolates is an inference that MDR strains are commoner within these two species, as each species has linkage to hyper-virulence, MDR-hv and outbreaks of carbapenem resistant Enterobacteriaceae, CRE [86-88] with similar scenarios in isolates of *Proteus mirabilis* and *Proteus vulgaris*. The presence of antimicrobial resistant, AMR Gram-negative bacteria in food sources creates palpable anxiety to the consuming public [89]. This contributes to the growth in the cumulative number of AMR bacterial strains and to the probability of mortality rates reaching 10 million deaths annually by the year 2050 [82,90], with most of the burden expected to be borne in poor income countries [91].

Conclusion.

Fresh raw vegetables on retail in the open market in Owo Township, Ondo State yielded bacterial counts much above the recommended upper limits, including microorganisms globally recognized in food-related outbreaks of illnesses, thus representing a source of health hazard to the consumer. It is therefore, expedient for the Public Health Departments to re-double efforts in providing appropriate education and guidelines for safe processing, handling, transportation and retail stages for vegetables and similar farm produce in Ondo State, Nigeria.

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