Int. J. Adv. Multidiscip. Res. (2024). 11(6): 63-73

International Journal of Advanced Multidisciplinary Research
ISSN: 2393-8870
www.ijarm.com
(A Peer Reviewed, Referred, Indexed and Open Access Journal)
DOI: 10.22192/ijamr Volume 11, Issue 6 -2024

Research Article

DOI: http://dx.doi.org/10.22192/ijamr.2024.11.06.007

Raw Green Vegetables: Microbial Contamination, Prevalence and Antibiotic Susceptibility Profile

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Keywords

Raw Vegetables, Microorganisms, Multidrug Resistance, Antibiotics, Prevalence

Abstract

Fresh vegetables are rich in nutrients and their contamination has been identified as a vehicle for animals and human foodborne illness.The present study investigated the microbial contamination of fresh *Telfairia occidentalis* (fluted pumpkin) and *Talinum triangulare* (waterleaf) and antibacterial susceptibility profile. Prevalence of bacteria and fungi was also determined. The vegetables were analyzed for the presence and levels of microorganisms using standard microbiological procedures. Antibiotic susceptibility was carried out using commercial antibiotic disc. Results revealed that bacterial and fungal counts of the vegetables from all the retail outlets exceeds recommended limit. Prevalent bacteria isolated from the vegetables include *Escherichia coli, Enterococcus faecalis, Salmonella typhimurium, Staphylococcus aureus, Bacillus cereu, Bacillus subtilis, Enterobacter cloaca* and *Shigella dysenteriae*.Five moulds isolated includes*Aspergillus flavus, Fusarium poae, Penicillium notatum, Rhizopus stolonifer*,*Mucor*sp and one yeasts, *Saccharomyces cerevisiae*.The medical significant of some of these bacterial isolates in food and feeds had been reported. The fungal isolates had been implicated in the production of mycotoxins and resultant mycotoxicosis in animals and humans. *Saccharomyces cerevisiae* is involved in fermentation and a few responsible for food deterioration and spoilage. All the bacterial isolates were resistant to penicillin, ampicillin and amoxicillin. 87.5% of the bacterial isolateswas sensitive to amikacin, 50% sensitive to ciprofloxacin and levofloxacin, 25% sensitive to erythromycin while 75% resistant to erythromycin. 50% of the isolates were also resistant to ciprofloxacin and levofloxacin, suggesting a multidrug resistant scenario.These results indicate that the vegetables are significantly contaminated and have poor microbiological quality that could potentially result in outbreak of foodborne illnesses. These findings highlight the importance of microbiological quality surveillance of fresh produce in formal and informal markets, as these products can be a reservoir of multidrug resistant bacteria harbouring antibiotic resistance and virulence genes potentially impacting human health.

Introduction

Leafy green vegetables are generally considered healthy; however, the increase in consumption of minimally processed, including ready-to-eat, leafy green vegetables increases the potential for human exposure to foodborne pathogenic bacteria through the food chain (Castro-Ibáñez *et al.,* 2015; Beshiru *et al.,* 2022). However, harvesting, preparation, processing, and distribution of leafy green vegetables have resulted in increased complexity in the supply chains and potential contamination with foodborne pathogenic bacteria (Mogren *et al.,* 2008), consequently, leafy green vegetables were ranked as the highest priority in terms of their microbiological hazard *(FAO, 2008). In addition,* resident microflora in the soil, application of nonresident micro flora via animal manures, sewage or irrigation water, transportation and handling by individual retailers are vehicles to contamination(Ray and Bhunia 2007).

Several outbreaks have highlighted fresh vegetables as potential vehicles of foodborne disease (Singh,2017; Popeda *et al*., 2018; Nüesch-Inderbinen *et al.,* 2016). Although not in outbreak proportions, *E. coli* illnesses have also been reported in Southern Nigeria (Afolayan, *et al.,* 2022; Odetoyin, *et al.,* 2022).

The emergence of multidrug-resistant organisms that have led to increased mortality and economic burden has increased almost exponentially. Global reports documenting the infection burden of common and diverse bacterial pathogens that have developed resistance to available antimicrobial agents is alarming (Vasconcelos *et al.,* 2016; Nikaido, 2009).

This study investigates microbial contaminants in fresh raw vegetables sold at major markets in Oweri, Imo State, Nigeria.

Materials and Methods

Sample collection

Fresh vegetables *Telfairia occidentalis* (fluted pumpkin) and *Talinum triangulare* (Waterleaf) were collected from three markets (FUTO Market, Eziobodo Market and Ihiagwa Market) in Owerri, Imo State, Nigeria. Samples were collected in sterile polythene zipper pouch and transported to the Anthonio Van Leuwenhoek Research Centre, Nekede, ImoState and analysed microbiologically. Sample size was determined by the method of Kothari (2004).

Preparation and inoculation of samples

Twenty grams (20 g) of the leaf samples were macerated in 180 ml of sterile distilled water in stomacher blender. One milliliter of the suspension was decimally diluted and 0.1 ml inoculated into freshly prepared surface dried bacteriological and mycological media (Cheesbrough, 2000; Sharma, 2000; Beishir, 1987).

Enumeration of Total Viable Counts and characterization of isolates

Colony counts obtained on the media were counted using a digital colony counter and expressed as colony forming units per gram (CFU/g) to obtain total population (Harrigan and McCance, 2000).

Microbial isolates were characterized based on cultural (colonial), microscopic and biochemical methods with reference to standard manuals. Yeasts and other yeast-like organisms were identified using staining technique as applicable to bacteria. Moulds were identified using the wet-mount method described as (Beishir, 1987). The identities of the isolates were cross-matched with reference to standard manuals for the identification of bacteria (Harrigan and McCance, 2000; Buchannan and Gibbon, 2000) and fungi (Barnett and Hunter, 1998).

Results

Table 1 shows the total viable counts of bacteria and fungi isolated on bacteriological and mycological Media. Bacteria grew luxuriantly on nutrient agar (2.0 x 10^6-1.37 x 10^7) and least on Eosin Methylene Blue Agar (0-1.8 x 10^6). Growth on Salmonella Shigella Agar (0-8.3 x 10^6) and Potato Dextrose Agar was also significant (1.2 x 10^5-2.48 x 10^6). Bacteria and fungi were isolated from the vegetables in all the markets under investigated (Table 1).

Colonial, microscopic and biochemical characteristics of bacterial isolate is shown in Table 2. Four Gram positive bacteria, namely, *Staphylococcus aureus, Bacillus cereus, Enterococcus faecalis Bacilus subtilis* and four Gram negative bacteria namely, *Salmonella typhimurium, Shigella dysentariae, Escherichia coli* and *Enterobacter cloaca* were identified. Five moulds, *Aspergillus flavus, Penicillium notatum, Fusarium poae, Rhizopus stolonifer, Mucor* species and one yeast, *Saccharomyces cerevisiae* were isolated from the vegetables (Table 3).

Table 1 Total Bacteria and Fungi Counts on Vegetables (n=50)

Samples/market	NA (Cfu/g)	PDA (Cfu/g)	EMBA (Cfu/g)	SSA (Cfu/g)
FUPM	1.11x 10^7- 2.11 x 10^7	5.2 x 10^6-2.24 x 10^7	0-1.0 x 10^6	8.3 10^3-1.11 x 10^4
FUWL	2.0 x 10^6-1.02 x 10^7	1.01 x 10^7-2.48 x 10^7	0-1.1 x 10^6	0-1.1 x 10^3
IHPM	2.0 x 10^6-6.1 x 10^6	1.2 x $10^6$4.1 x 10^6	1.2 x 10^6-3.0 x 10^6	1.7 x 10^3- 2.9 x 10^3
IHWL	1.37 x 10^7-2.33 x 10^7	1.55 x 10^7-2.77 x 10^7	0-2.1 x 10^6	3.0 x 10^3-6.6 x 10^3
EZPM	7.9 x 10^6-9.1 x 10^6	2.0 x 10-3.3 x 10^6	1.0 x 10^6-2.1 x 10^6	4.0 x 10^3-8.7 x 10^3
EZWL	8.4 x $10^6$1.09x 10^7	8.0 x $10^6$1.78 x 10^7	1.8 x 10^6-2.2 x 10^6	7.0 x 10^3-1.00 x 10^4

FUPM, FUTO Pumpkin; FUWL, FUTO, Waterleaf; IH PM, Ihiagwa pumpkin; IHWL, Ihiagwa waterleaf; EZPM, Eziobodo pumpkin; EZWL, Eziobodo waterleaf, NA, Nutrient agar;PDA, Potato dextrose agar; EMBA, Eosin methylene blue agar; SSA, Salmonella shihella agar.

Table 2 Colonial, Microscopic and Biochemical Characterization of Bacterial Isolates

Colonial and biochemical characteristics	Spore Formation	Motility	Gram morphology	Capsule production	Identity of isolates
Small smooth moist and shiny yellow colonies on mannitol salt agar, catalase and coagulase positive, indole negative. Ferment glucose, lactose and sucrose	-	-	+S	-	*Staphylococcus aureus*
Greenish metallic sheen on eosin methylene blue agar, catalase, indole positive, coagulase and oxidase negative. Ferment lactose, glucose and sucrose.	-	+	-R	-	*Escherichia coli*
Small smooth moist and shiny cream colonies on nutrient agar, catalase, oxidase,coagulase, indole negative, citrate and Voges Proskaeur positive. Ferment glucose, sucrose and lactose.	-	-	+S	-	*Enterococcus faecalis*
Dull and dry serrated flat colonies on nutrient agar, catalase, Voges Proskaeur and citrate positive. Oxidase, coagulase, indole negative. Ferment glucose	+	+	+R	-	*Bacillus cereus*
Large mucoid raised pink colonies on MacConkey agar. Catalase and citrate positive, oxidase, coagulase and indole negative. Ferment lactose and glucose.	-	+	-R	-	*Enterobacter cloaca*
Small circular moist and shiny black fish eye colonies on salmonella shigella agar. Catalase, citrate, nitrate, and hydrogen sulphide positive. Ferment glucose, maltose and mannitol.	-	+	-R	-	*Salmonella typhumirium*
Small smooth moist and shiny light pink colonies on salmonella shigella agar. Catalase, oxidase, coagulase, indole negative, nitrate positive. Ferment maltose.	-	-	-R	-	*Shigella dysenteriae*
Rough, mucoid and slimy cream colonies. Catalase, citrate, nitrate positive. Oxidase, coagulase, indole negative. Ferment glucose, sucrose and mannitol.	+	+	+R	-	*Bacillus subtilis*

S, spherical shaped; R, rod shaped; +, positive; -, negative

Table 3 Colonial and Microscopic Characteristics of Fungal Isolates on Waterleaf and Pumpkin Leaf

Colonial Characteristics	Microscopic Characteristics	Identity of isolates
Tall fluffy filamentous hyphae bearing black spores at the tip	Hyphae non septate. Spores enclosed in a sporangium	*Rhizopus stolonifer*
Short white hyphae bearing black spores at its tip	Hyphae septate. Conidia globosed bearing sterigma	*Aspergillus flavus*
Dirty green powdery spores enclosed in white hyphae	Septate hyphae. Conidia arranged like mop-head	*Penicillium notatum*
Large mucoid and raised/domed shaped cream colonies	Gram positive spherical and oval budding cells	*Saccharomyces cerevisiae*
Short fluffy cotton wool like hyphae	Hyphae non septate. Spores enclosed in sporangium on septate sporangiophore.	Mucor sp
Fluffy cotton wool like hyphae with red at the reverse/background	Conidia arranged like sickle cell. Hyphae septate.	*Fusarium poae*

The prevalence of the bacteria and fungi isolated from the vegetable in the different market is shown in Table 4. Bacteria and fungi are prevalent in the order Ihiagwa market > Eziobodo market> FUTO market. This trend is also applicable to the two vegetables. Percentage occurrence of bacterial and fungal isolates is shown in Table 5. *Enterococcus faecalis* (28.6%) is highest in percentage while *Escherichia coli*, *Salmonella typhumurium*, *Shigella dysentariae* and *Enterobacter cloaca*were the least with 2.4%. Others are *Staphylococcus aureus* (14.3), *Bacillus subtilis* (21.4) and *Bacillus cereus* (26.2). The percentage occurrence of *Aspergillus flavus* and *Rhizopus stolonifer* is 25.0% each, while that of *Saccharomyces cerevisiae*, *Penicillium notatum*, *Fusarium poae* and *Mucor* sp is 12.5% each.

Table 4 Prevalence of Bacteria and Fungi isolated from Waterleaf and Pumpkin leafacross the Markets

Samples	Bacterial isolates (%)	Fungal isolates (%)
FUPM	12	9
FUWL	15	5
IHPM	33	12
IHWL	41	17
EZPM	28	12
EZWL	31	8

Table 5 Percentage occurrence of Bacteria and Fungi in waterleaf and pumpkin leaf

Bacterial isolates	% occurrence	Fungal isolates	% occurrence
Staphylococcus aureus	6(14.3)	*Rhizopus stolonifer*	2(25.0)
Escherichia coli	1(2.4)	*Aspergillus flavus*	2(25.0)
Enterococcus faecalis	12(28.6)	*Penicillium notatum*	1(12.5)
Bacillus cereus	11(26.2)	*Saccharomyces cerevisiae*	1(12.5)
Enterobacter cloaca	1(2.4)	Mucor sp	1(12.5)
Salmonella typhumirium	1(2.4)	*Fusarium poae*	1(12.5)
Shigella dysenteriae	1(2.4)		
Bacillus subtilis	9(21.4)		
Total	100		100

Number in parenthesis represent percentage

Distribution of bacteria and fungi isolated from pumpkin leaf and waterleaf across the markets is shown in Table 6. Bacteria is most prominent in waterleaf and pumpkin obtained from FUTO and Eziobodo market respectively. *S. aureus, B. subtilis, Ent. faecalis, B.cereus* was isolated from all the vegetables in all the markets. *Sal. typhimurium, Sh. dysentariae* was isolated from waterleaf obtained from FUTO market, whereas, *E. coli* and *E. cloaca* was isolated from pumpkin in Eziobodo market.

Saccharomyces cerevisiae was isolated from the vegetables in all the markets. *Fusarium poae* and *Mucor* sp was isolated from pumpkin leaf in Ihiagwa and waterleaf in Eziobodo market respectively. *Penicillium notatum* was isolated from pumpkin leaf obtained from FUTO market. *Aspergillus flavus* and *Rhizopus stolonifer* were isolated from the vegetables in five markets. Antibiotic susceptibility test of the bacterial isolates on commercial antibiotic is shown in Table 7. All the bacterial isolates were resistant to penicillin, ampicillin and amoxicillin. 87.5% of the bacterial isolates weresensitive to amikacin, 50% sensitive to ciprofloxacin and levofloxacin, 25% is sensitive to erythromycin whereas 75% is resistant to erythromycin. 50% of the isolates were also resistant to ciprofloxacin and levofloxacin.

Staphylococcus aureus is resistant to all the antibiotics. *Shigella dysentariae*, *Enterococcus cloaca*, *Escherichia coli* and *Salmonella typhymurium* is sensitive to amikacin, ciprofloxacin and levofloxacin. *Bacillus cereus* is resistant to all the antibiotics except amikacin, whereas *Bacillus subtilis* is sensitive to amikacin and erythromycin. *Enterococcus faecalis* is sensitive only to amikacin and erythromycin and resistant to others.

Table 6 Distribution of Bacterial and Fungal Isolates in Waterleaf and Pumpkin leaf

Sample Location	Bacterial isolates	Fungal isolates
FUPM	*S. auteus, B. subtilis, Ent. faecalis, B.cereus*	*Saccharomyces cerevisiae, Penicillium notatum, Aspergillus flavus, Saccharomyces cerevisiae*
FUWL	*S. auteus, Sal. typhimurium, Sh. dysenteriae, B. subtilis, Ent. faecalis, B.cereus*	*Aspergillus flavus, Rhizopus stolonifer, Saccharomyces cerevisiae*
IHPM	*S. auteus, B. subtilis, Ent. faecalis, B.cereus*	*Mucor* sp, *Aspergillus flavus, Saccharomyces cerevisiae*
IHWL	*S. auteus, B. subtilis, Ent. faecalis, B.cereus*	*Saccharomyces cerevisiae, Rhizopus stolonifer*
EZPM	*S. auteus, E. coli, B. subtilis, Ent. faecalis, B.cereus, E. cloaca*	*Saccharomyces cerevisiae, Aspergillus* sp
EZWL	*S. auteus, B. subtilis, Ent. faecalis, B.cereus*	*Rhizopus stolonifer, Fusarium poae, Saccharomyces cerevisiae*

Table 7 Zone of Inhibition of Bacterial Isolated on Commercial Antibiotics (mm)

Bacterial isolates	AK 30 µg	CL 5µg	E 15µg	AMC 30µg	G 10 µg	AMP 10 µg	LO 25 µg
B. cereus	15	0	0	0	0	0	0
B. subtilis	25	0	34	0	0	0	0
Sh. dysenteriae	19	18	0	0	0	0	20
E. coli	20	34	0	0	0	0	20
S. aureus	0	0	0	0	0	0	0
E. cloaca	18	34	0	0	0	0	32
Ent. faecalis	19	0	15	0	0	0	0
Sal. Typhimurium	14	30	0	0	0	0	34

AK=Amikacin; CL=Ciprofloxacin; E=Erythromycin; AMC=Amoxycilin; G=Penicillin; AMP=Ampicillin; LO=Levofloxacin

Discussion

The consumption of fresh vegetables purchased from market places where hygiene practices for food product handling are unregulated exposes consumers to foodborne bacteria, some of which may be antibiotic-resistant (Holzel *et al.*, 2018). This study presents results on the microbiological quality and occurrence of multidrug resistant (MDR) microorganisms (bacteria and fungi) from fresh vegetables sold by retailers and street vendors in three major markets in Owerri, Imo State, Nigeria.

Results obtained for viable counts of bacteria and fungi isolated from the vegetables suggested gross contamination and counts far above recommended standard (WHO, 2015; WHO, 2010). The microbiological quality of fresh produce, mainly leafy greens, sold at different markets have been studied (Holzel *et al.*, 2018; Kuan *et al.*, 2017; Bernstein *et al.*, 2017).Leafy greens have previously been prioritized as the highest level of concern in terms of fresh produce safety from a global perspective (Center for Food Safety, 2014; Health Protection Agency, 2009)

Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Enterobacter cloaca, Salmonella typhymurium, Shigella dysentariae, Bacillus subtilis and *Bacillus cereus* were identified as bacteria, whereas *Saccharomyces cerevisiae, Rhizopus stolonifer, Aspergillus flavus, Penicillium notatum* and *Mucor* species were identified as fungi in this study.*Aspergillus, Penicillium and Fusarium* species had been reported to produce mycotoxins responsible for mycotoxicosis in various foods and feeds (Jay *et al.*,2005; Frazier and Westhoff, 2003). *Rhizopus* and *Mucor* species have also been reported to produce potent mycotoxins (Jay *et al.*,2005; Frazier and Westhoff, 2003). *Saccharomyces cevevisiae* are mostly involved in food fermentation although some cause food deterioration and spoilage at low pH (Savard *et al.*, 2002). Most of the isolates had been reported as major contaminants of vegetables (Popeda, 2018; Singh,2017; Nüesch-Inderbinen *et al.*, 2016; Eni *et al.*, 2010). *Escherichia coli* and *Salmonella* species have been particularly implicated in foodborne disease outbreaks associated with consumption of vegetables (Maffei *et al.*, 2013).

The wide distribution and percentage occurrence of these organisms in the vegetables is a pointer to a major contamination. However, isolation of these bacteria from vegetables evaluated in this study should raise food safety concerns among consumers, vendors, vegetable farmers, tourists, and health authorities because of their implications in global foodborne disease outbreaks (Scallan *et al.,* 2011).

However, spoilage of vegetables could occur if not properly washed before stored (CDC, 2016). Eni *et al.*(2010) reported that bacterial contamination of vegetables decreased significantly when vegetables were washed with increasing concentrations of vinegar. This suggests that the consequence of disease outbreaks that usually occurs as a result of the consumption of contaminated vegetables can be effectively managed if vegetables are properly handled from both pre- and post-harvest processes.

However, the presence of these bacteria and fungi on the vegetables may be attributed to some cultivation practices, including irrigation of vegetable farms with contaminated water, application of animal wastes as manure to enrich the soil, pre- and post-harvest handling practices, transportation, storage and processing (Eni *et al.,* 2010). It is well established that most vegetable farmers in Nigeria commonly use pipe-borne water, water from streams, wells and storm drains to irrigate their vegetable farms. Microorganisms detected in this study are reported also in other studies where water from streams, wells and storm drains were confirmed to have been contaminated with similar microorganisms (Abakari *et al.,* 2018; Akoachere *et al.,* 2018).

Maffei *et al.*(2013) reported 95% loss of fluted pumpkin to the presence of fungi. Leaf spot disease usually affect the leaves which are the main sources of assimilate thereby, reducing the photosynthetic area available for the plants. The occurrence of leaf spot in the fluted pumpkin usually leads to leaf defoliation and a reduction in leaves available for harvest and sale commercially. Combined fungal and bacterial activities often lead to deterioration and eventual spoilage of vegetables, making them unwholesome for consumption and potential sources of foodborne illnesses.

Most of the bacterial isolates are multidrug resistant. Amikacin is the only drug that was able to inhibit 87.5% of the bacterial isolates. The zone of inhibition is a crucial parameter in assessing the effectiveness of antibiotics against multi-drug resistant bacteria. The zone of inhibition refers to the area around antibiotic discs or wells on an agar plate where bacterial growth is inhibited. This was substantiated by the observation of Kumar and Jangir (2017) who have observed similar results and suggested that access to clean water, health education to sellers and proper disposal of waste materials are important to improve the quality of food.

Conclusion

The effectiveness of antibiotic treatment of diseases will decline due to the development of antibiotic resistance in the environment. It is evident from the present results that multiple antibiotic-resistant bacteria can thrive on surfaces of food products especially vegetables. This can serve as an environmental reservoir and can therefore provide a route to multidrug-resistant pathogens to enter human population. The study of resistance in the environmental bacteria can help in guiding the development of strategies to counteract this resistance. From the study, it can be concluded that to detect any changing patterns, there is a need to monitor antibiotic sensitivity at regular intervals and knowledge of local presence of bacterial organisms and antibiotic sensitivities and not universal guidelines should provide the direction of antibiotic selection.

This study found out that contamination may occurred during both pre- and post-harvest processes. Produce can be contaminated at any point in time from the farm to the retail markets; pre-harvest via untreated manure, untreated irrigation water, contaminated soil; post-harvest during handling at the farm, transportation and storage, and even during display in retail markets. The increasing amount of antibiotics used in animal feeds and as pesticides affects entire agro-ecosystems, and threatens raw vegetable production systems, the soil ecosystem and the quality of groundwater. Unsafe or contaminated water used for washing, spraying and dipping also contaminates vegetables during post-harvest processing, which needs to be regulated by monitoring and implementing good agricultural practices. Re-use of water for washing and contaminated soil particles can also cause cross-contamination. Poor personal hygiene by vendors and agricultural workers, poor sanitation facilities, and unhygienic conditions at marketplaces are also associated with the contamination of fresh vegetables and fruits with multi-drug resistant bacteria. Some types of vegetables in the retail markets may be more likely to be contaminated with antibiotic-resistant bacteria due to constant handling by shoppers to check freshness or ripeness, although no evidence exists to prove this mechanism.

Recommendation

The presence of antibiotic resistant bacteria on retailed vegetable portend public health risks of unknown magnitude. Preventing this contamination exposure through fresh produce may be challenging considering the cross-cutting issues related to food security and food safety, accentuating the need for a holistic approach encompassing pre-harvest and post-harvest processing and the distribution of produce. Good farming practices and processing of post- harvest produce remains the sin qua non to maintaining wholesome and healthy vegetables that meets the consumers satisfaction.

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DOI:10.22192/ijamr.2024.11.06.007	

How to cite this article:
Azuwike, C.O., Justice-Alucho, C. H., Okeke, O. D., Ihejirika, C. E, Kalu, U. C and Braide, W. (2024). Raw Green Vegetables: Microbial Contamination, Prevalence and Antibiotic Susceptibility Profile. Int. J. Adv. Multidiscip. Res. 11(6): 63-73.
DOI: http://dx.doi.org/10.22192/ijamr.2024.11.06.007