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Research Article

Antibiotic Susceptibility of Microorganisms and Proximate Profiles of Minimally Processed Leafy Vegetables Purchased from a University Campus

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ABSTRACT

The study assessed the antibiotic susceptibility of microorganisms and proximate profiles of minimally processed leafy vegetables (*Telfairia occidentalis* and *Ocimum gratissimum*). The vegetable samples were processed by washing and blanching and analysed for microbiological and proximate parameters using standard protocols. Comparatively, blanched samples had lower microbial load than washed samples. Range of mean values of microbial counts of processed samples was; Aerobic plate count 2.15 - $2.50x10^3$CFU/g; Coliform 0.00 - $8.45x10^3$CFU/g; Salmonella-Shigella 1.20 - $2.05x10^3$CFU/g and fungal count 1.20 -$5.50x10^2$CFU/g. These values are below permissible limits for foodstuffs by Food and Agriculture Organization (FAO)/World Health Organization (WHO). Identified microbes and relative abundance (%) were *Staphylococcus* sp. (25.8), *Bacillus* and *Vibrio* species (22.6), *Escherichia coli* (9.7), *Klebsiella* and *Shigella* species (6.5) and *Proteus* and *Salmonella* species (3.2) and *Alternaria* sp (31), *Fusarium* and Yeast species (24.1) and *Penicillium* sp (20.7). Proximate composition indicated increased moisture and crude carbohydrate but decreased crude protein, fibre and fat contents after blanching. Susceptibility profile showed that all the Gram-negative bacteria were 100% resistant to Nalidixic acid and Gram positives, 100% resistant to Streptomycin and Amoxil with multiple antibiotic resistance index (MARI) ranging from 0.1-1.0. Seven species including *Proteus, Shigella* and *Staphylococcus* depicted high degree of multi-drug resistance (MDR) and five exhibited 83% resistance to Ciproflox and Septrin. *Vibrio* and *Bacillus* species were the most susceptible to several drugs with MARI of ≤ 0.2. The incidence of Enterobacteriaceae, *Staphylococcus, Bacillus* and fungi portends serious health risk to consumers. Therefore, to ensure food safety, good agricultural and food processing practices, personal hygiene, and environmental sanitation monitoring are required of all stakeholders and consumers to minimize outbreak of vegetable-borne diseases.

Keywords: leafy vegetables, microorganisms, multi-drug resistance, proximate profile, food safety

Introduction

Leafy vegetables or greens are plant leaves eaten as a vegetable accompanied by tender petioles and/or shoot. When consumed raw in a salad leafy vegetables can be called 'salad greens'. They are often harvested from short-lived shrubs or herbaceous plants such as Pumpkin, lettuce, spinach, African basil (Scent leaf), etc. Leafy vegetables that have undergone physical alteration from its original state (by washing, cutting, slicing, shredding, blanching, etc) but remains fresh and convenient for consumers are considered minimally processed. Blanching is a process in which vegetables are briefly exposed to boiling water prior to low temperature storage and serves as a necessary step to maintain vegetable quality, inactivates enzymes, reduces the microbial load and extend shelf life (Olorode *et al*., 2015). Blanching involve three basic processes; boiling, steaming and boiling + sodium bicarbonate (Titi Mutiara *et al*., 2013; Severini *et al*., 2016). Reports indicate that both the textural and microbiological qualities of processed vegetables are

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influenced not only by variety and maturity at harvest but by processing such as blanching and storage conditions (Yixiang *et al*., 2012).

Daily consumption of fresh fruits and vegetables are a source of nourishment and an integral part of a healthy and balanced diet due to their high nutritional value (Darmon *et al*., 2005). Green vegetables provide one of the most important human diet that contains carbohydrates, proteins, vitamins, minerals and fibre (Brett *et al.*, 1996). Their role in reducing the risk of lifestyle associated illnesses such as heart disease, diabetes and cancer has accentuated desirability and consumption whilst low intake has been estimated to cause about 31% of ischemic heart disease and 11% of stroke globally (Sagar and Suresh, 2010).

Telfairia occidentalis and *Ocimum gratissimum* are common vegetables used singly or in combination in preparing a variety of dishes in Nigeria. *T. occidentalis* is one of the most cherished herbaceous vegetables used in the preparation of soups for nutritional, as blood tonic by locals for medicinal functions and treatments of diabetes, reducing the risk of cardiovascular diseases, colon cancer, lowers plasma cholesterol, etc (Herrera *et al*., 2009; Lennox and Nkra, 2016). *O. gratissimum* is an aromatic shrub abundant in Nigeria and provides a range of culinary and medical applications; as food, flavourant and for treatment of high fever, cholera, diarrhoea, sunstroke and as an antimicrobial (Berendsen *et al*., 2015; Adebolu and Salau, 2005). It also has antimicrobial, antispasmodic, antiseptic and antimalarial properties. However, the fact that these green leafy vegetables are eaten minimally processed to retain nutrient, green colour as well as other structural architecture calls for precaution as viable pathogens may still abound. Improper handling during/after post-harvest processing such as cutting or slicing, shredding, washing, transporting, packaging, distribution and storage can also create opportunities for microbial cross-contamination (Rahman *et al*., 2022). A variety of microorganisms are associated with the contamination of leafy vegetables, mushrooms and mustard cress such as members of the Enterobacteriaceae, *Pseudomonas putida*, *Bacillus cereus*, *Staphylococcus aureus*, *Aeromonas hydrophila*. *Aspergillus niger*, *A. flavus*, *Rhizopus stolonifer*, *Mucor micheli*, *Alternaria alternate*, *R. oligoporus* and *Candida albicans*, due to activities and processes involved from cultivation to post-harvest operations (Al-kharousi *et al*., 2016; Jega *et al*., 2021). Enterobacteriaceae are opportunistic pathogens linked to human infections, such as urinary tract infections, septicaemia (Sivakumar *et al*., 2020) as well as gastroenteritis.

Most of these microbes have become drug resistant due to frequent exposure and abuse of antibiotics. The occurrence of multidrug-resistant Enterobacteriaceae has become a global public health challenge. Antibiotic resistance via mechanisms like mutational changes or acquisition of resistance genes through horizontal gene transfer from other bacteria or phages in different environments coupled with the abuse of beta-lactams and fluoroquinolones as common therapeutic choice for Enterobacteriaceae infections in animals and humans have been reported in literature (Munita and Arias, 2016; Beshiru *et al*., 2023). Multidrug-resistant (MDR) infections are more hazardous than infections caused by bacterial pathogens that are not resistant to multiple drugs because they have been found tremendously difficult to treat (Koilybayeva *et al*., 2023).

Some toxigenic producing species of *Staphylococcus* and MDR *E. coli* and *Salmonella* as well as opportunistic microbes have been reportedly linked to raw vegetables as potential reservoir of food-borne disease outbreaks in several countries (Rahman *et al.*, 2022; CDC, 2022). This indicates that fresh vegetables can be contaminated with bacterial pathogens at multiple points throughout its production and supply chain. With no formal food-borne disease monitoring system in Nigeria, infection of food origin is endemic, with an estimated cost of $3 billion and an estimated 17–25% of total illnesses (Oludare *et al*., 2016) and local health system recorded an estimated 25% mortality.

Raw consumption of fresh leafy and non-leafy vegetables and fruits results in the exposure of humans to foodborne bacterial pathogens, including antibiotic-resistant bacteria (ARB) (Holzel *et al.*, 2018; Founou *et al*. 2021) as well as MDR strains. Generally, most vendors of RTE vegetables are not monitored by food safety/regulatory agencies and this accentuates hazard of contamination with infectious agents. Based on established facts, contaminants of leafy vegetables which on ingestion has the capacity to cause infection in the consumers has necessitated actionable policy on the administration of proper antibiotic chemotherapy.

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These reasons prompted attempts to explore such avenues and bridge the information gap by determination of antibiotic susceptibility of microorganisms and proximate profiles of minimally processed leafy vegetables purchased in a University campus.

Materials and Methods

Collection of leafy vegetable samples

Fresh samples of leafy vegetables of Fluted Pumpkin and Scent leaves were purchased from subsistence farmers within the Rivers State University (RSU) main campus, Nkpolu-Oroworukwo, Port Harcourt.

Purchased samples were put into sterile polythene bags and transported to the Microbiology Laboratory of the University where they were identified by a Plant taxonomist as *Telfairia occidentalis* and *Ocimum gratissimum* respectively.

Plate 1: Pumpkin (*T. occidentalis*)

Plate 2: Scent leaves (*O. gratissimum*)

Processing of leaf samples

Sorting and washing of the leafy vegetables

A total of 4-sterile polyethylene bags (2 of Pumpkin and Scent leaves respectively) containing a sufficient mass (500g) of leaves for processing were inspected for fungal growth and other contaminants and the size, colour and appearance were sorted. Defective, insect-infested and contaminated leaves were discarded. Approximately 150g of fresh leaves with a healthy appearance was selected and about 20g of these leaves was removed and placed in a sterile Petri dish as control.

The remaining 130g of leaves were washed under running tap water for 1min to remove soil and other dirt and subsequently rinsed in distilled water. Washing is not only used to remove field soil, residual debris and surface microorganisms but to remove fungicides and insecticides/pesticides from leafy vegetables.

Blanching

This is a special heat treatment to inactivate enzymes such as catalase and peroxidase present in vegetables, where the leaves are submerged in boiling water 85-100°C (hot water blanching) for 3minutes. These minimally processed leafy vegetable samples were divided into appropriate portions of 20g each from both washed and blanched and subjected to microbiological and proximate analyses.

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Microbiological Analysis

Enumeration and isolation of isolates

Tenfold serial dilution was carried out for the enumeration and isolation of bacteria from the samples. A 25gram of the sample was weighed into 225ml of sterile normal saline to obtain the homogenate. From which decimal dilutions of 10^{-1} to 10^{-3} were made. An aliquot of 0.1ml of appropriate dilution of the samples were spread-plated in duplicate on surface-dried solidified nutrient agar (NA; Titan Biotech, India) for the enumeration of total heterotrophic bacterial count (THBC) after incubation at 37°C for 24hours. Eosin methylene blue and MacConkey agar (MA; Titan Biotech, India) were used for the enumeration of total coliform count (TCC) as well as *Escherichia coli* (EC). *Salmonella-Shigella* agar (SSA) was used to determine *Salmonella-Shigella* count (SSC) whereas Thiosulphate citrate bile salt sucrose agar (TCBS). Sabouraud Dextrose Agar (SDA) was used for isolation and enumeration of fungal count (FC). The plates were incubated at 37ºC for 24 hours and colonies that developed were counted and recorded as colony forming units per gram (CFU/G).

Characterization and Identification of bacterial and fungi Isolates

Discrete colonies were picked based on their cultural, morphology, microscopic and macroscopic examination and biochemical tests. The isolates were subcultured on solid NA and SDA and subsequently on slants of the respective agar media and persevered at refrigeration temperature. Identification of the isolates as bacteria and fungi (Tables 2 and 3) was carried out as described in (Sneath *et al.,* 1986; Cheesbrough, 2006).

Susceptibility Assay of the Bacteria Isolates

Antimicrobial sensitivity test was performed using Kirby-Bauer method to measure the ability of an antibiotic to inhibit bacteria growth *in vitro* by disc diffusion (CLSI, 2020). The pure cultures of bacteria isolated were aseptically inoculated into 5ml of sterile peptone water and incubated at 37°C for 18-24hours. A turbid suspension of the isolates was made in distilled water using 0.5 McFarland Standard prepared as a comparator. A sterile swab was dipped into the bacteria suspension, pressed on the side of the test tubes to allow excess drip off and then evenly smeared on the entire surface of the Mueller Hinton agar. Sterile forceps was then used to position the commercial multiple antibiotic discs (Optun Laboratories Nig., Ltd, Tables 4 and 5) on the medium containing each of the test bacteria and incubated at 37ºC for 24hours and zones of inhibition were measured. Antibiotic profile of the isolates whether susceptible, intermediate or resistant was determined via an established interpretative chart (CLSI, 2020). Multidrug resistance (MDR) and multiple antibiotic resistance index (MARI) of the isolates were determined. MDR = Resistance to ≥1 antimicrobial in ≥3 antimicrobial class. MARI= Number of antimicrobial an organism was resistant to / Total number of antimicrobial the organism was subjected to.

Determination of Proximate Composition of leafy vegetables

Proximate composition for moisture contents, Crude protein, fibre, fat, ash and carbohydrate of the samples were determined using AOAC (2012).

Statistical Analysis

Statistical analysis was conducted using 2 software programs described below. Geometric mean, standard deviation, ranges and median were calculated using Microsoft Office Excel 2013 (Microsoft, USA) and one-way ANOVA test with Tukey comparisons to derive statistical differences ($p < 0.05$) of microbial levels by using SPSS version 21 (IBM, USA).

Results

The mean microbial counts of minimally processed Pumpkin leaf (*Telfaire occidentalis*) and Scent leaf (*Ocimum gratissimum*) are as presented in Table 1. The data indicate that the microbial counts of *O. gratissimum* were lower than that those of *T. occidentalis.* The highest microbial counts for total aerobic heterotrophic count (THBC) occurred in washed Scent leaves (WSL) was $2.50\pm0.69^{a}x10^{3}$CFU/g for total Coliform count (TCC) ($8.45 \pm 0.60^{b}x10^{3}$) in washed Pumpkin leaf WP, 2.05 ± 0.92^{a} $x10^{3}$ for total *Salmonella-Shigella* count (TSSC) in WSL, 4.60 ± 2.81^{b} $x10^{3}$ CFU/g in WSL for total *Vibrio* count (TVC) and total fungal count (TFC) 5.50 ± 3.08^{b} $x10^{2}$CFU/g were observed in WP. Generally,

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blanched leafy vegetables (Pumpkin and/or Scent leaves) depicted the lowest microbial counts (Table 1).

Table 1: Mean Microbial Counts (x10^3 CFU/g) of Washed and Blanched Vegetables (Pumpkin and Scent leave)

Microbes	WP	BP	WSL	BSL	P-value
Total Heterotrophic Bacteria	2.40 ± 0.57^a	2.15 ± 0.08^a	2.50 ± 0.69^a	2.41 ± 0.12^a	0.923
Total Coliform	8.45 ± 0.60^b	0.00 ± 0.00^a	3.00 ± 0.28^c	2.85 ± 0.21^c	0.001
Salmonella Shigella	1.31 ± 0.63^a	1.20 ± 0.08^a	2.05 ± 0.92^a	1.75 ± 0.35^a	0.510
Total Vibrio	2.00 ± 1.41^a	1.23 ± 0.04^a	4.60 ± 2.81^b	2.88 ± 1.73^a	0.650
Total Fungi (x10^2 CFU/g)	5.50 ± 3.08^b	1.32 ± 0.11^a	1.94 ± 0.08^a	1.20 ± 0.14^a	0.408

Legend: WP=Washed Pumpkin; BP=Blanched Pumpkin; WSL=Washed Scent Leaf; BSL=Blanched Scent Leaf; CFU/g=Colony forming unit per gram. ^{a,b,c} = Means with the same alphabet across the row shows no significant difference (p≥ 0.05).

The percentage occurrence of bacterial isolates from washed and blanched leafy vegetables are presented in Table 2. *Bacillus* and *Staphylococcus* species had the highest percentage occurrence in both washed (WP) and blanched (BP) Pumpkin (*T. occidentalis*) whereas *Vibrio* sp was the highest in washed Scent leaf (WSL) (*O. gratissimum*) followed by *Bacillus* and *Staphylococcus* species. However, after blanching (BSL) of (*O. gratissimum*) *Vibrio* sp still predominated followed by *E.coli* and *Staphylococcus* species.

Table 2: Percentage Occurrence of Bacterial Isolates from Washed and Blanched Leafy Vegetables

Bacterium	***Telfaira occidentalis***				***Ocimum gratissimum***			
	WP	**(%)**	**BP**	**(%)**	**WSL**	**(%)**	**BSL**	**(%)**
Bacillus sp	3	(30)	2	(40)	2	(16.66)	0	(0)
Escherichia. coli	1	(10)	0	(0)	1	(8.33)	1	(25)
Klebsiella sp	1	(10)	0	(0)	1	(8.33)	1	(25)
Proteus sp	0	(0)	0	(0)	1	(8.33)	0	(0)
Salmonella sp	0	(0)	0	(0)	1	(8.33)	0	(0)
Shigella sp	1	(10)	0	(0)	1	(8.33)	0	(0)
Staphylococcus sp	3	(30)	2	(40)	2	(16.66)	1	(25)
Vibrio sp	1	(10)	1	(20)	3	(24.99)	2	(50)
Total	**10**		**5**		**12**		**4**	

Legend: WP=Washed Pumpkin; BP=Blanched Pumpkin; WSL=Washed Scent Leaf; BSL=Blanched Scent Leaf. The number (s) in parentheses are in percentage.

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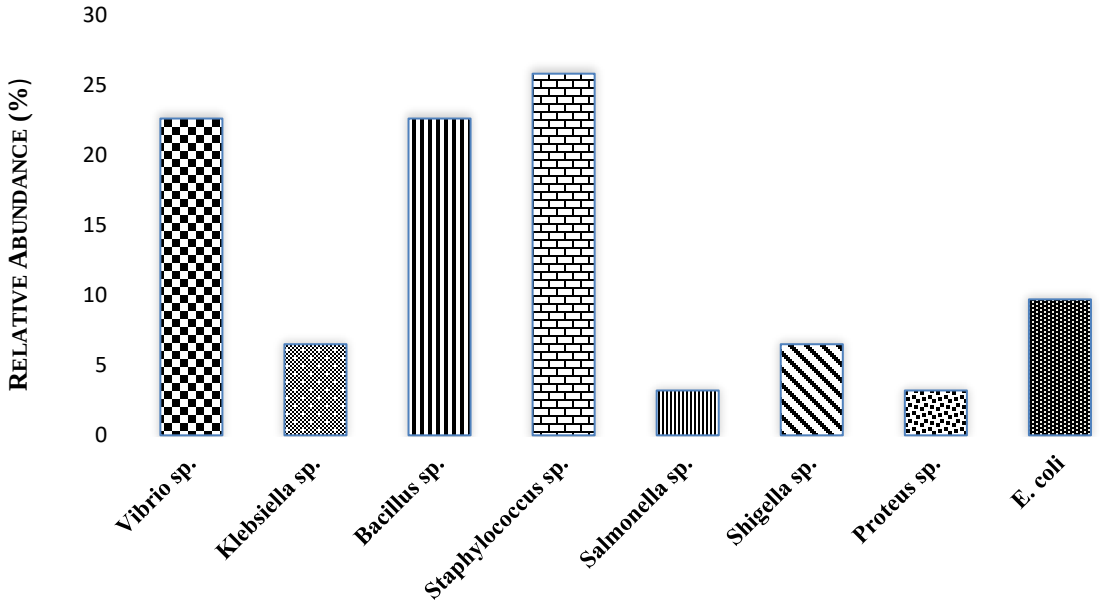

Figure 1: Relative abundance (%) of bacterial isolates from washed and blanched leafy vegetables

Relative abundance (%) of bacterial isolates from both minimally processed leafy vegetables are depicted in Figure 1. The most abundant being *Staphylococcus* species (25.8%) followed by *Bacillus* and *Vibrio* species (22.6%) and *E. coli* (9.7%) whereas the least are *Salmonella* and *Proteus* species (3.2%) respectively.

Table 3, presents the percentage occurrence of fungal isolates from minimally processed leafy vegetables. *Fusarium* species had the highest percentage occurrence (42.86%) followed by Yeast species (28.57%) in WP whilst only Yeast species occurred in BP. On the other hand, *Alternaria* species predominated in both WSL and BSL with 35.71% and 42.86% respectively. BSL samples had *Alternaria* sp (42.86%) as the highest percentage occurrence followed by *Penicillium* and Yeast species (28.57%) respectively. Generally, the washed leafy vegetables had the highest percentage occurrence of fungal load than the blanched.

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Table 3: Occurrence (%) of fungal Isolates from Washed and Blanched Pumpkin and Scent Leaves

Fungi	WP	(%)	BP	(%)	WSL	(%)	BSL	(%)
Alternaria sp	1	(14.29)	0	(0.00)	5	(35.71)	3	(42.86)
Fusarium sp	3	(42.86)	0	(0.00)	4	(28.57)	0	(0.00)
Penicillium sp	1	(14.86)	0	(0.00)	3	(21.43)	2	(28.57)
Yeast sp	2	(28.57)	1	(100)	2	(14.29)	2	(28.57)
Total	**7**		**1**		**14**		**7**	

Legend: WP=Washed Pumpkin; BP=Blanched Pumpkin; WSL=Washed Scent Leaf; BSL=Blanched Scent Leaf. The numbers (s) in parentheses are in percentage

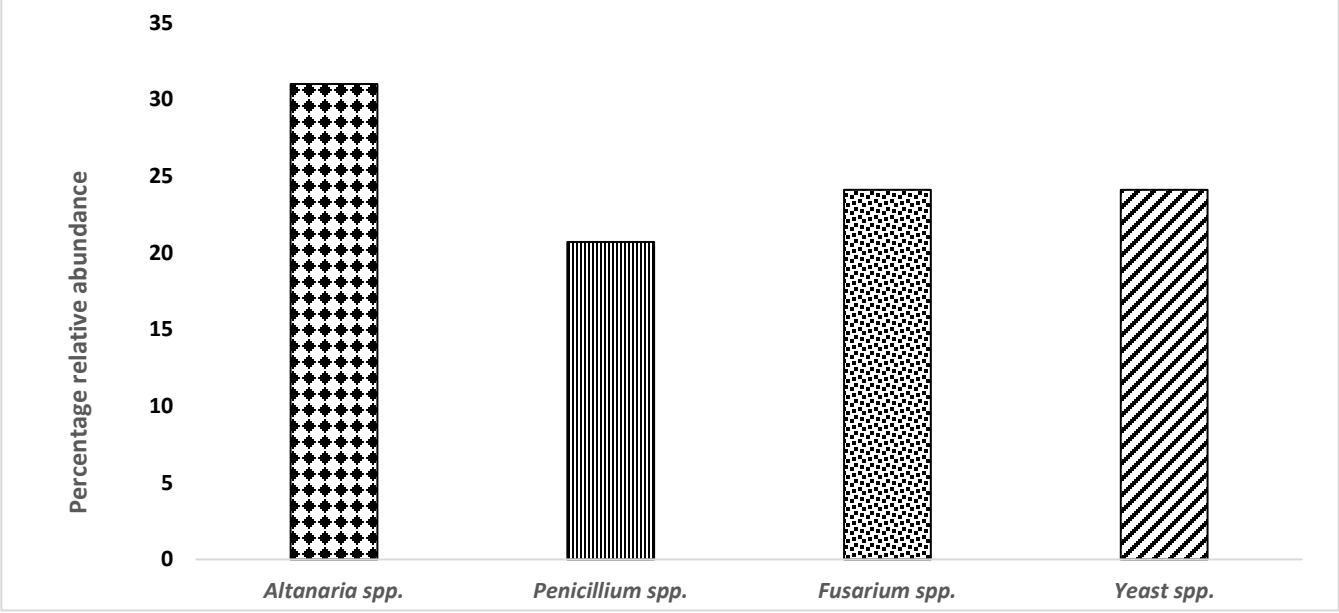

Figure 2. Percentage relative abundance of fungal isolates from washed and blanched leafy vegetables

The trend of percentage relative abundance (PRA) of fungal isolates in leafy vegetables are depicted in Figure 2. *Alternaria* sp, had highest PRA (31.0%) followed by *Fusarium* and Yeast (24.1%) respectively. *Penicillium* species was the least (20.7%).

Antibacterial susceptibility pattern of 6 Gram negative isolates and MAR index are presented in Table 4. *Proteus* and *Shigella* species exhibited resistance to all the antibiotics followed by *Salmonella* species and *E. coli*. These elevated levels of resistance may be accountable for the high MAR index, >0.2 of most of the species. All the bacterial isolates showed 100% resistance to Nalidixic acid (NA), 5 species exhibited 83% resistance to Ciproflox and Septrin and others levels of resistance (in %). *Vibrio* sp was the most susceptible to all the drugs except Nalidixic acid (NA)

hence it has an MARI of < 0.2. *Escherichia coli*, and two genera; *Klebsiella* and *Vibrio* showed 50% susceptibility to Reflacin and Ceporex whereas others were < 50%. Five bacteria; *E.coli*, *Proteus* sp, *Salmonella*, *Klebsiella* and *Shigella* species were multi-drug resistant (MDR).

Table 4: Antibacterial Susceptibility of 6 Gram Negative Isolates to the 10 Antibiotics

Antibiotic class	*E. coli*	*Proteus*	*Salmonella*	*Klebsiella*	*Shigella*	*Vibrio*	No. R (%)	No. S (%)
Aminoglycosides								
Gentamicin (30µg) GN	S	R	R	R	R	S	4(67)	2(33)
Streptomycin (30µg) S	S	R	I	R	R	S	4(67)	1(17)
Quinolones								
Ciprofloxacin (30µg) CPX	R	R	R	R	R	S	5(83)	1(17)
Tarivid (10µg) TRD	R	R	R	S	R	S	4(67)	2(33)
Reflacine (10) REF	S	R	R	S	R	S	3(50)	3(50)
Nalidixic acid (10µg) NA	R	R	R	R	R	R	6(100)	0(00)
Septrin (25µg) S	R	R	R	R	R	S	5(83)	1(17)
Cepores (10µg) CEP	S	R	R	S	R	S	3(50)	5(50)
Augmentin	R	R	R	S	R	S	4(67)	2(33)
Penicillin								
Ampicillin (10µg) Amp	R	R	R	S	R	S	4(67)	2(33)
MARI	0.7	1.0	0.9	0.5	1.0	0.1		

Phenotype showing MDR and number of drugs *7(50-100), *10(50-100), *9(50-100), *5(67-100), and *10(50-100) for the first five bacteria above.

* = Number of drugs; Number in parenthesis in (%)

Legend: MARI=Multiple antibiotic resistance index; MDR = Multi-drug resistance. R=Resistance; S=Susceptibility; I=Intermediate.

Displayed in Table 5, is the antibacterial susceptibility profile and MARI of two Gram positive isolates exposed to antibiotics. *Staphylococcus* species showed resistance to all the drugs with an MARI of 1.0. The two bacterial species were 100% resistant to Streptomycin (S) and Amoxil (AML). *Bacillus* species exhibited 50% susceptibility to all drugs except Streptomycin and Amoxil with MARI of 0.2. Of the two Gram positive bacteria *Staphylococcus* species showed MDR to ten drugs.

Table 5. Antibacterial susceptibility of 2 Gram positive isolates to the 10 antibiotics

Antibiotic class	***Staphylococcus* sp**	***Bacillus* sp**	**No. R (%)**	**No. S (%)**
Aminoglycosides				
Streptomycin (30µg) S	R	R	2(100)	0(00)
Gentamicin (30µg) GN	R	S	1(50)	1(50)
Fluoroquinolones				
Ciprofloxacin (30µg) CPX	R	S	1(50)	1(50)
Norfloxacin (20µg) NB	R	S	1(50)	1(50)
Levofloxacin (10 µg) LEV	R	S	1(50)	1(50)
Phenicols				
Chloramphenicol (20µg) CH	R	S	1(50)	1(50)
Penicillin				
Amoxil (30µg) AML	R	R	2(100)	0(00)
Ampiclox (30µg) APX	R	S	1(50)	1(50)
Macrolides				
Erythromycin (30µg) E	R	S	1(50)	1(50)
Antimycobacterial				
Rifampicin (30 µg) RD	R	S	1(50)	1(50)
MARI	**1.0**	**0.2**		

Phenotype showing **MDR** and number of drugs *10(50-100) for *Staphylococcus* sp.
* = Number of drugs; Number in parenthesis in (%)
Legend: MARI=Multi-antibiotic resistance index; MDR = Multi-drug resistance.

The mean values of proximate composition of minimally processed leafy vegetables are displayed in Table 6. Moisture contents and crude carbohydrate increased in blanched samples but much so in BSL upto 84.79 ± 0.29^{b} and 4.51 ± 0.11^{c} respectively. Conversely, there was decrease in crude protein, fibre and fat mean values after blanching. Ash content increased in BP (4.49 ± 0.06^{a}) but decreased in BSL

(1.20±0.11[a]) which is indicative of substantial availability of minerals in BP than in BSL.

Table 6: Mean Value of Proximate composition of washed and blanched leafy vegetable (on dry weight basis g/100g)

Parameter	WP	BP	WSL	BSL	P-value
Moisture content	82.75±0.35[ab]	83.34±0.94[ab]	82.00±1.41[a]	84.79±0.29[b]	0.124
Crude protein	7.29±0.00[d]	5.07±0.06[c]	2.84±0.11[b]	2.54±0.00[a]	0.000
Ash	1.89±0.13[a]	4.49±0.06[a]	1.95±0.21[b]	1.20±0.11[a]	0.015
Carbohydrate	0.43±0.13[a]	2.49±0.46[b]	4.13±0.41[c]	4.51±0.11[c]	0.001
Crude fibre	7.42±0.05[c]	6.94±0.06[b]	7.95±0.19[d]	6.48±0.00[a]	0.001
Fat	0.32±0.13[a]	0.49±0.17[a]	0.59±0.02[a]	0.41±0.01[a]	0.210

Legend: WP =Washed Pumpkin leaf, BP= Blanched Pumpkin leaf, WSL= Washed Scent leaf, BSL=Blanched Scent leaf,

[a-d] = Means with same superscript across the column shows no significant difference (p≥0.05)

Discussion

The leafy vegetable samples of both *T. occidentalis* and *O. gratissimum* harboured relatively high initial microbial load. The isolates were predominantly Gram negative bacteria (GNB) and Gram positive bacteria (GPB) and few of fungi dominated by *Alternaria* species. This phenomenon which resulted in the dominance of GNB and GPB may be due to their ability to survive the brief heat processing treatment, post processing contamination and cell-wall architecture particularly for GPB. In addition, it has been reported that endospores of *Bacillus* are more resistant than the vegetative cells to harsh weather conditions and even to antimicrobial treatments (Codex, 2007). Similar microflora and ewtrend had been earlier reported in Nigeria and Ethiopia (Jega *et al*., 2021; Biniam and Mogessie, 2010) and in other parts of the globe (Zhao *et al*., 2017). Lower percentage occurrence of isolates after blanching may be attributable to intolerance to thermal processing condition and cell wall components. With the initial microbial load, reduction of counts by minimal processing (washing and blanching) may still leave some proportion of microorganisms unaffected but relatively lower than the permissible limits of 10^6CFU/g for total viable count, 10^4CFU/g for fungi and 10^3CFU/g of coliforms as stipulated (FAO/WHO, 2004). However, there is need for effective processing at every step from planting to consumption of leafy vegetables, in order to reduce microbial contamination which might eventually lead to food poisoning and food spoilage.

Even, after these treatments both vegetables in most cases are cooked (as traditionally practiced) which results in further reduction in microbial load and may consequently impact the nutritional contents prior to consumption. The load of Enterobacteriaceae, *Staphylococcus*, *Bacillus* and *Vibrio* species as well as *Alternaria*, *Fusarium* species and yeasts as revealed in this study conforms with findings by other investigators who also reported these isolates as opportunistic and common contaminants of leafy vegetables (Beshiru *et al*., 2023; Lennox and Nkra, 2016). Higher bacterial counts than fungal may be attributed to the alkaline nature (higher pH) of leafy vegetables and faster growth of the former than the latter (fungi) even in conditions that favour both (Karim and Wee, 1996). Therefore, the relatively high percentage of *Staphylococcus*, *Bacillus*, *Vibrio* and *E.coli* as well as *Fusarium* species in fresh vegetables could be hazardous to the consumer's health as most of these are capable of producing food borne toxins.

In Nigeria, 200,000 people die of food poisoning each year, according to the National Agency for Food and Drug Administration and Control (Onyeaka *et al*., 2021) and requires emergency to monitor proliferation, toxins and resistant traits in microorganisms. Resistance strategies of bacteria to broad spectrum of

antibiotics has become a global health challenge especially among GNB. Bacteria use two major genetic strategies to adapt to the antibiotic "attack", such as mutations in gene(s) often associated with the mechanism of action of the compound and acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer
(HGT) from other bacteria or phages in different environment (Munita and Arias, 2016).

In fact, the World Health Organization has named antibiotic resistance as one of the three most important public health threats of the 21st century (WHO, 2014). *Proteus* and *Shigella* species exhibited resistance to all the antibiotics which may have accounted for the high MARI of 1.0 and 100% resistance to Nalidixic acid (NA) and five of the GNB showed MDR of (50-100%) to 5-10 antibiotics which was slightly higher than the range obtained by previous workers (Lateef, 2004). This may be linked to the fact that most therapeutic choice for Enterobacteriaceae infections in animals and humans are β-lactams and fluoroquinolones due to low toxicity, high potency and abuse which would have contributed to the phenomenon of resistance in Nigeria, Ghana and other parts of the globe (Owusu *et al*., 2023; Obeng-Nkrumah *et al*., 2013). In addition, the plasmids conveying determinants coding Extended-spectrum β-lactamases (ESBLs) also harbour other determinants conferring resistance to aminoglycosides and fluoroquinolones (Zhao *et al*., 2022). The relatively high level of resistance to antimicrobial agents has been reported as a reflection of misuse or abuse of these agents in the environment (Silva and Hoffer 1993). Resistance of *E.coli*, *Salmonella*, *Shigella* and *Proteus* species to β-lactams especially Ampicillin as discovered in this study coincides with observations made by other workers (Biniam and Mogessie., 2010; Owusu *et al*., 2023). The resistance of *E.coli* and *klebsiella* species to most of the quinolones (CPX, TRD, NA and S) corroborates report of other researchers (Anejo-Okopi *et al*., 2017). *Klebsiella* species has been reported to cause urinary tract infections with an increasing rate of drug resistance to many commonly used antibiotics (Caneiras *et al.*, 2019; Jafari *et al.*, 2019). Their ability to utilize different resistance mechanisms to counteract the effects of antibiotics has been attributed to the production of destructive enzymes, target alteration, efflux pumps and porin loss (Caneiras *et al.*, 2019; Odewale *et al*., 2023). The susceptibility of *Vibrio* species to all the drugs may be due to recent exposure to these antibiotics.
Additionally, many intrinsic resistance mechanisms, such as target alteration, decreased permeability, and efflux, can take place in the same cell at the same time, resulting in fluoroquinolones and other antibiotic resistance at a high degree. *Staphylococcus* sp exhibited 100% resistance to β–lactams (Amoxil and Ampicillin) a phenomenon earlier reported (Amadi, *et al*., 2022; Lateef, 2004). Such outcomes were attributed to the elevated resistance to the drugs (Goldberg *et al*., 2010) whereas *Bacillus* demonstrated 50% susceptibility to 8 antibiotics, except Streptomycin and Amoxil with MARI of 0.2 which may be associated with recent exposure. Resistance of *Staphylococcus* sp to NB, CH, and GN respectively had also been reported and such results were attributed to drug abuse (Ugbogu *et al.*, 2007; Buck *et al*., 2005). MARI of >2.0 predicts high risk source of contamination and frequent exposure to antibiotics. Multiple drug-resistance is an extremely serious public health problem and it has been found associated with the outbreak of major epidemics throughout the world. High prevalence of MDR indicates a serious need for broad-based, local antimicrobial surveillance and planning effective interventions to reduce MDR in such pathogens (Sandhu *et al*., 2016; Magiorakos *et al*. 2012). Understanding the numerous pathways for antibiotic resistance spread via vegetable and salad microbiomes is crucial for mitigating drug resistance.

Proximate composition mean values of both washed and blanched *T. occidentalis* and *O. gratissimum* increased in Moisture and carbohydrate contents but within the range of those reported (Bangash *et al.*, 2010) whereas other parameters decreased with processing. The decrease in protein contents may be due to denaturation after blanching which negates the report of Jega *et al.*, (2021). The results also indicate that Pumpkin contains substantial amounts of protein and ash and less of fat than Scent leaf with or without blanching and agrees with the earlier work (Jega *et al*., 2021). Both vegetables contain approximately equal amounts of crude fibre irrespective of processing treatments. The crude fibre content in the studied vegetables showed slight variation. The difference in crude fibre contents may be due to soil fertility and age of leaves at the time of harvest as previously reported (Uusiku *et al*., 2010). This study, however, have demonstrated that minimal processing impacted the microbial load and improved or retained the

freshness and nutritional values of the leafy vegetables.

Conclusion

Fresh leafy vegetables harbour a variety of microorganisms such as Enterobacteriaceae, *Staphylococcus* and *Bacillus* species as well as *Alternaria*, *Fusarium*, *Penicillium* species and yeasts. Most of these organisms are opportunistic and have been associated with vegetable-borne diseases especially when they reached infective dose. The microbial load reported on *T. occidentalis* and *O. gratissimum* are below the permissible limits recommended by food regulatory agencies and may not constitute biohazard to consumers. Furthermore, the microbial load of both leafy vegetables decreased after blanching, suggestive of better treatment option(s) in terms of safety. However, the high levels of MARI and MDR bacteria on these samples underscores the need to monitor abuse of antibiotics, sanitation, processing and handling to ensure food safety and security to prevent foodborne illnesses.

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