African Journal of Environment and Natural Science Research
ISSN: 2689-9434
Volume 8, Issue 1, 2025 (pp. 236-257)
www.abjournals.org

PREVALENCE OF COLIFORMS AND THEIR MULTIDRUG-RESISTANT STRAINS IN SOIL AMENDED WITH POULTRY MANURE AND IN THE LEAVES OF FLUTED PUMPKIN DURING HARVEST AND IN MARKETS

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Cite this article:

Osula, A. O., Imade, S. O., Okwu, M. U. (2025), Prevalence of Coliforms and Their Multidrug-Resistant Strains in Soil Amended with Poultry Manure and in the Leaves of Fluted Pumpkin during Harvest and in Markets. African Journal of Environment and Natural Science Research 8(1), 236-257. DOI: 10.52589/AJENSR-YHDPSYTB

Manuscript History

Received: 11 Feb 2025

Accepted: 18 Mar 2025

Published: 8 Apr 2025

ABSTRACT: *The increase in antibiotic-resistant bacteria on food and vegetables has raised growing global concerns regarding food safety, particularly because the consumption of vegetables represents a direct exposure pathway for humans to soil bacteria. This research aimed to evaluate how poultry manured-soil and fluted pumpkins serve as vectors and reservoirs for coliforms and their multidrug-resistant strains. The study was conducted during the 2024 growing season at two fluted pumpkin farms and two markets in Benin City, Edo State, Nigeria. Coliforms were isolated using the spread plate technique. The isolates were identified using standard phenotypic methods and 16S rRNA gene sequencing. The Kirby-Bauer disk diffusion method was employed to assess the susceptibility of the isolates to antibiotics from seven distinct classes. Isolates exhibiting resistance to antibiotics from at least three antibiotic classes were classified as multidrug-resistant. Klebsiella. pneumoniae, Escherichia coli, Proteus vulgaris, and Enterobacter cloacae were detected in all analyzed samples of fluted pumpkin leaves and soil, while Citrobacter freundii was exclusively found in market samples. Coliforms showed strong resistance to amoxicillin and cefotaxime, and moderate resistance to other antibiotics. Multidrug-resistant coliforms were identified in both soils and leaves, with highest multiple antibiotics resistance (MAR) indices in farm soils. The lowest MAR indices were in fluted pumpkin leaves from New-Benin and Oba Markets. The research concluded on the importance of continuous monitoring of antibiotic resistance patterns in coliforms and their multidrug-resistant strains to ensure food safety, while recommending appropriate decontamination of fluted pumpkins or other agricultural produce before consumption.*

KEYWORDS: Fluted pumpkin, Coliforms, Multidrug-resistant, *Klebsiella*, *Escherichia.*

INTRODUCTION

The growing use of poultry manure in vegetable farming is raising concerns about the spread of antimicrobial-resistant bacteria and their genes into humans, and this poses a major threat to public health [1]. Antibiotic-resistance genes (ARGs) may transfer from bacteria to harmful pathogens through the food chain, aided by mobile genetic elements like integrons, plasmids, and transposons. With high levels of transmissible mobile genetic elements in animal manures, these can assist the horizontal movement of ARGs between bacteria in manure and those naturally found in the soil [2]. Thus, manured soil is a reservoir for ARGs and mobile genetic elements, increasing the risk of antimicrobial resistance spreading into farmland and our food supply. Vegetables—including seeds, roots, fruits, stems, and leaves—have different soil contacts and exposure to environmental factors like rain and sun, which can affect their contamination levels at harvest. Although vegetables are essential for a healthy diet, the high prevalence of antibiotic-resistant bacteria from manured soils, especially on raw or minimally processed veggies, is concerning. This can expose consumers to soil microorganisms and contribute to the spread of antimicrobial resistance. Ready-to-eat vegetables are often contaminated with pathogens like *Escherichia coli*, *Salmonella*, *Shigella*, *Listeria monocytogenes*, *Clostridium perfringens*, *Bacillus* spp., and enterococci [3]. While washing vegetables can reduce the microbial load, it may not eliminate all contaminants, leaving consumers potentially exposed to harmful bacteria [4]. In Nigeria, poor poultry farming practices include using antibiotics in low doses for growth promotion [5]. This misuse can lead to the development of multidrug-resistant (MDR) bacteria, which may be spread through manure used as fertilizer [6]. What's more? Recommended farming practices—such as preventing produce contact with pathogens in raw manure, composting manure before use, and allowing time between manure application and planting—are often overlooked. Farmers in Benin City, Edo State, commonly apply poultry manure directly to soil for growing vegetables. This research aims to investigate how poultry manure-amended soil impacts the cultivation of fluted pumpkin leaves and their potential role in carrying and spreading multidrug-resistant coliforms in Benin City, Edo State, Nigeria.

MATERIALS AND METHODS

Geographic Description of the Study Area

Benin City is situated at latitude 6°19' E to 6°21' E and longitude 5°34' E to 5°44' E, with an average elevation of 77.8 meters above sea level. This city boasts a rich history, as it was once the capital of the now-defunct Bendel State and currently is the capital of Edo State. The area is situated within Nigeria's humid tropical rainforest belt. Here, the rainy season typically kicks off in March or April and wraps up around October or November. Rainfall can be quite intense, often with two peaks, and there is a brief dry spell in August known colloquially as the 'August Break.' Geologically speaking, the terrain is characterized by a top layer of reddish soil, made up of weathered clay sand. Numerous historical moats surround various parts of the region, and it has been described as a cultivated plain extending southwestward. Borehole records indicate substantial deep chemical weathering over time. The soil profile predominantly consists of reddish-brown sandy laterite, with intermittent layers of porous sandy clay that can extend to important depths, as observed in the region's boreholes. Three key river systems flow through the Benin Region: the Ikpoba River, the Ogba River, and the Owigie-Ogbovben River

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African Journal of Environment and Natural Science Research
ISSN: 2689-9434
Volume 8, Issue 1, 2025 (pp. 236-257)
www.abjournals.org

systems. The exact urban location of the farms involved in this study is illustrated in Figure 1. The two fluted pumpkin farms are located on the outskirts of the Isiohor community, which is part of the Ovia North-East Local Government Area in Edo State. One market included in this study, New-Benin market, is found in Egor Local Government Area, while another, Oba market, is situated in Oredo Local Government Area of Edo State.

Study Design

The research was conducted in Benin City, Edo State, Nigeria, during the 2024 cropping season in two farms and two markets. This study examined the prevalence and characteristics of coliforms found in soil and fluted pumpkin leaves. Prior to the present study, the farm areas had not been cropped, and had not been irrigated or received any manure amendments. The farms were not located near any poultry farms within a 2-kilometer radius. Beds, each 3 meters by 3 meters, were leveled to a gentle slope and positioned 1 meter apart. Poultry manure from Benin City was collected and aged for at least three weeks on farms before use. Two weeks before planting, nine kilograms of cured poultry manure were applied to each of the nine square meter seed beds, with the manure being uniformly incorporated using a hoe. The planting pattern involved placing two seeds per hole, with a spacing of 75 cm by 75 cm. Harvesting of leafy shoots occurred three times, at intervals of two weeks, between 6 and 10 weeks after planting.

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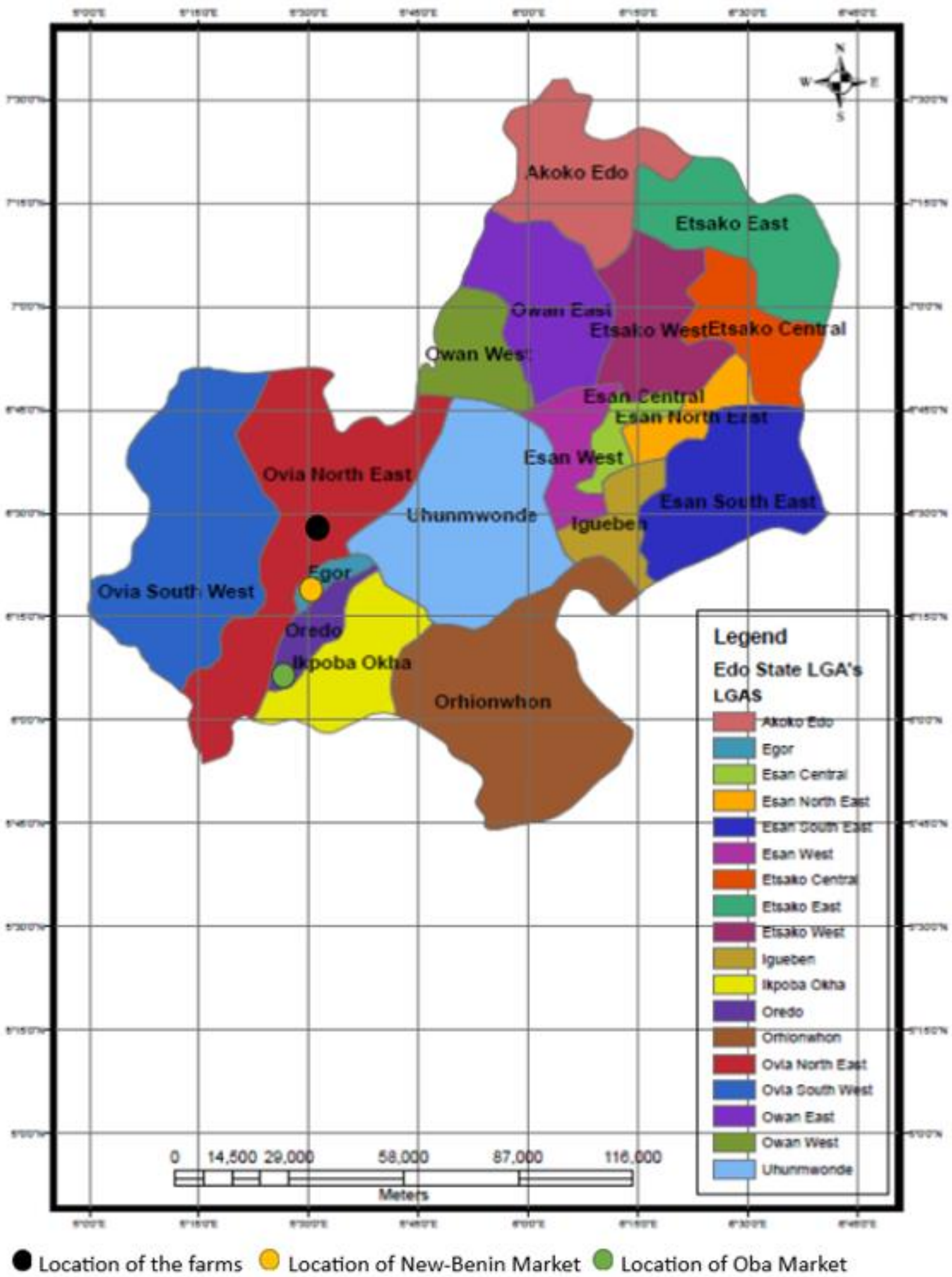

Figure 1: Map of Study Locations

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Soil and Vegetable Sampling

Thirty (30) soil samples were taken randomly, to a depth of 15 cm, from six poultry manure-fertilized seed beds in each of the farms on day 0 (the day of manure incorporation into the soil before planting) and at harvest (days 42, 56 and 70). Following the random sampling, the samples from poultry manure-fertilized seed beds were combined into a sterile polyethylene bag and thoroughly mixed. Soil samples from the farms were collected and transported to the lab in a cooler with ice packs for microbiological analysis. Similarly, 70 leaf samples were taken randomly from the poultry manure-fertilized seed beds on each of the harvesting days 42, 56 and 70, bulked into a sterile polyethylene bag, and kept in a cooler on ice packs for transport to the laboratory. Three sets of leaf samples were gathered from the farms. During each of the harvesting days 42, 56 and 70, thirty (30) raw fluted pumpkin leaf samples were randomly collected from each of the two different local open markets (Oba Market and New-Benin Market) in Benin City, separately bulked into a sterile polyethylene bag, and transported to the laboratory in a cooler with cool packs. Three (3) sets of leaf samples were gathered from the markets.

Isolation and Enumeration of Coliforms

The presumptive isolation and enumeration of coliforms was performed with the spread plate method [7]. Twenty-five-gram portion of each of the soil and fluted pumpkin leaf samples was separately mixed with 225 ml of 1.5% W/V sterile peptone water using a sterile glass jar blender. The mixture was then used to prepare serial dilutions up to 10^{-6}. One hundred microliters (100 µl) aliquots of each of the undiluted and serially diluted samples were spread on the surfaces of duplicate MacConkey agar Petri dishes, and were incubated at 35^{o}C for 24 hours. After incubation, bacterial colonies on the Petri plates were presumptively identified as coliforms, and were counted. The colony counts on the MacConkey agar Petri plates were subsequently used to deduce the presumptive total coliform counts (*pTCC*), expressed as colony-forming units per gram (CFU/g) of the sample.

Genus-level Identification of Coliforms Using Phenotypic Techniques

Phenotypic identification of the bacterial isolates was carried out using standard methods [8, 9]. The phenotypic tests carried out included bacterial colony examination, Gram-stain, catalase, oxidase, urease, indole, citrate, methyl red, Voges-Proskauer and triple sugar iron (TSI).

Species-level Identification of Coliforms Using Molecular Techniques

Species-level identification utilized a method that included polymerase chain reaction and partial sequencing of the 16S rRNA gene of the coliform bacterial isolates [10, 11]. The template DNA was extracted using the Zymo-Spin column according to the guidelines provided by the manufacturer (Zymo Research Corporation, USA). The highly purified template DNA from the coliform isolates was utilized to conduct the polymerase chain reaction (PCR). Universal 16S rRNA bacterial primers [27F-AGAGTTTGATCMTGGCTCAG; 1492R- GGTTACCTTGTTACGACTT], commonly utilized forbacterial classification, were applied to identify the existence of the 16S rRNA gene [10].

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The PCR procedure was performed using a 50 µl reaction mixture that included 2 µL of template DNA, 10mM Tris-HCL (pH 8.3), 50mM KCl, 2 mM MgCl2, 200 mM of each deoxynucleoside triphosphate (dNTPs) (Fermentas Inc., USA), 2 U of GoTaq Hot Start Polymerase (Promega, USA), and 0.5 µM of each primer. Amplification occurred on a GeneAmp PCR system 9700 (Applied Biosystems) using these cycling conditions: an initial denaturation at 95°C for 2 minutes, then 40 cycles that included denaturation at 94°C for 45 seconds; annealing at 55°C for 60 seconds; extension at 72°C for 120 seconds; and a final extension at 72°C for 300 seconds. Ten microliters of the amplified product were examined by gel electrophoresis using a 2% agarose gel made in Tris-Borate-EDTA buffer with 0.5 µg/ml of ethidium bromide at 100 V for 1 hour. The DNA band in the gel was then seen and recorded using the gel documentation system (Applied Biosystems). A molecular marker (100 base pair ladder) was processed simultaneously. DNA sequencing of the amplicon was performed using the dideoxy-chain termination technique [12]. The amplicon was purified using ExoSAP-IT (ThermoFisher Scientific). The purified amplicon was subsequently analyzed through cycle sequencing employing the Big Dye Terminator version 3.1 (Applied Biosystems) with standard cycling parameters. The purified cycle sequencing product underwent separation via capillary electrophoresis on an ABI 3730×I DNA analyzer. The sequence was subsequently quality assessed and proofread using Sequencher version 4.10.1 (Gene Codes Corporation, USA). The classification of coliforms was validated by comparing the experimentally obtained nucleotide sequence with the sequence database (rRNA_typestrains/prokaryotic_16S_ribosomal_RNA).

The comparison of sequences was conducted using the BLASTN 2.8.0 + software [National Center for Biotechnology Information (NCBI)]. The identity of a bacterial species was verified when the query 16S rRNA sequence of the coliform isolate aligned with the reference (subject) 16S rRNA sequence of a bacterial species in GenBank regarding sequence similarity and sequence identity. The coliform strains' sequences from the samples were submitted to the United States NCBI GenBank with various accession numbers.

Antibiotic Susceptibility Testing of the Coliforms

The coliform colonies underwent testing for multidrug resistance through the Kirby Bauer disc diffusion method as recommended by the Clinical and Laboratory Standards Institute [13]. A saline suspension of each pure bacterial culture, incubated and adjusted to 0.5 McFarland turbidity standards, was inoculated onto Petri dishes containing Mueller–Hinton agar, where antibiotic discs were positioned on the surface of the agar. Following 16–18 hours of incubation at 35°C, the diameter of the inhibitory zones surrounding each bacterial colony was classified as sensitive, intermediate, or resistant according to the interpretive standards for zone diameter set by the Clinical and Laboratory Standards Institute. *E. coli* ATCC 25922 served as a reference strain to identify any possible mistakes in the antibiotic disc diffusion susceptibility test. A total of 10 antibiotic discs from seven distinct antibiotic classes were evaluated. The classes of antibiotics included beta-lactam, beta-lactam-beta-lactamase inhibitor, fluoroquinolone, aminoglycoside, sulfonamide, macrolide, and phenicol. The antibiotics discs tested included gentamicin (30 µg), imipenem (10 µg), meropenem (10 µg), amoxicillin (30 µg), amoxicillin-clavulanic acid (30 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), cotrimoxazole (25 µg), erythromycin (15 µg), and chloramphenicol (30 µg). The coliform isolate was

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considered multidrug-resistant if it showed resistance to antibiotics from at least three distinct classes of antibiotics.

Estimation of Multiple Antibiotic Resistance Indices (MAR)

MAR was conducted following previously outlined methods [14] to assess the risk of obtaining multidrug-resistant coliforms from agricultural soil and in the harvested fluted pumpkin leaves both at the farm and at the market. MAR was determined by utilizing Equation 1. A MAR value exceeding 0.20 suggested a significant risk of obtaining multidrug-resistant coliforms from the sampled sites.

$$\text{MAR} = \frac{\sum(AR)}{A \times B} \qquad (1)$$

MAR is the mean multiple antibiotic resistance index. *AR* is the antibiotic resistance scores of each coliform colony, defined as the sum of antibiotic classes to which a particular coliform colony exhibited resistance. *A* is the total number of antibiotic classes tested. *B* is total count of coliform colonies examined.

Calculation of the Confirmed (Actual) Total Coliform Counts

The confirmed total coliform counts (*cTCC*) in each of the samples was deduced with Equation 2.

$$cTCC = H \times pTCC \qquad (2)$$

pTCC is the presumptive total coliform count. *H* is the prevalence of confirmed coliforms.

$$H = \frac{\text{Number of presumptive coliform colonies that were confirmed as actaul coliforms}}{\text{Total number of presumptive coliform colonies examined}} \qquad (3)$$

The counts of multidrug-resistant coliforms (*MRCC*) in each of the samples was deduced with Equation 4.

$$MRCC = K \times cTCC \qquad (4)$$

cTCC is the confirmed total coliform count. *K* is the prevalence of multidrug-resistant coliforms, deduced with Equation 5

$$K = \frac{\text{Number of confirmed coliform colonies that were multidrug-resistant}}{\text{Total number of confirmed coliform colonies examined}} \qquad (5)$$

Statistical Analysis

Descriptive statistics of coliform counts and frequency datasets were done with the Number Cruncher Statistical Software (NCSS) version 12. Levene test of homogeneity, Shapiro-Wilk test, Kruskal-Wallis nonparametric one-way ANOVA test and correlations were also performed with the NCSS software. The test of the hypothesis was considered statistically significant if the achieved level of significance (p) was less than 0.05.

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Volume 8, Issue 1, 2025 (pp. 236-257)
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RESULTS

Identified Coliforms

Tables 1 to 3 show the taxonomic characterization of coliforms isolated from the farm soil, harvested fluted pumpkin leaves at the farm and fluted pumpkin leaves sold in markets. As shown in Table 1, *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus vulgaris*, and *Enterobacter cloacae* were isolated from both farm soil (Farms 1 and 2). *Salmonella enterica* was found only in one of the farm soils. *E. coli* was the most abundant coliform in soil from farms 1 and 2, followed by *K. pneumoniae* and *E. cloacae*. The least abundant coliforms were *S. enterica* and *P. vulgaris* in the soil of Farms 1 and 2, respectively. Representative *E. coli* strain IM-OS01FS1, *S. enterica* strain IM-OS02FS1, *K. pneumoniae* strain IM-OS03FS1, *P. vulgaris* strain IM- OS04FS1, and *E. cloacae* strain IM-OS05FS1 isolated from the soil of Farm 1 have been deposited in the United States National Center for Biotechnological Information (NCBI) GenBank database under accession numbers PV012514, PV012515, PV012516, PV012517 and PV012518. Similarly, representative isolates of *K. pneumoniae* strain IM-OS06FS2, *P. vulgaris* strain IM-OS07FS2, *E. coli* strain IM-OS08FS2, and *E. cloacae* strain IM-OS09FS2 from the soil of Farm 2 were deposited in the NCBI GenBank database under accession numbers PV012519, PV012520, PV012521 and PV012522.

The coliforms isolated from harvested fluted pumpkin leaves in Farms 1 and 2 were similar to those isolated from the farm soil (Table 2). *E. coli* was the most abundant coliform in the fluted pumpkin leaves from Farms 1 and 2, followed by *E. cloacae* and *K. pneumoniae*. The least abundant coliforms were *S. enterica* and *P. vulgaris* in fluted pumpkin leaves harvested from Farms 1 and 2, respectively. Representative *S.* enterica strain IM-OS10PF1, *E.*

Table 1: Taxonomic Characterization of Coliform Isolates in the Farm Soil

Sampled farms	Rep. isolates from soil	Colonial and morphological characteristics		Biochemical characterizations												16S rRNA sequence homology		Identified coliforms	Frequency of occurrence of coliforms
		Growth on the MA Petri plates	Gram staining	Ca	Ox	Ur	Ci	In	Mr	Vp	Triple Sugar Iron test					16S similarity	16S identity		Farm 1 [F (%), Q = 120] Farm 2 [F (%), Q = 126]
											G	L	S	Ga	HS				
Farm 1	1	Pink colony	Negative rods	+	-	-	-	+	+	-	+	+	+	+	-	99.41%	98.41%	*Escherichia coli*	52 (43.33%)
	2	Colorless colony	Negative rods	+	-	-	+	-	+	-	+	-	-	+	+	100.00%	99.73%	*Salmonella enterica*	2 (1.67%)
	3	Pink colony	Negative rods	+	+	-	+	-	-	+	+	+	+	+	-	100.00%	99.51%	*Klebsiella pneumoniae*	40 (33.33%)
	4	Colorless colony	Negative rods	+	-	+	+	-	+	-	+	-	-	+	+	99.52%	99.05%	*Proteus vulgaris*	8 (6.67%)
	5	Pink colony	Negative rods	+	-	+	+	-	-	+	+	-	+	+	-	100.00%	99.78%	*Enterobacter cloacae*	18 (15.00%)
Farm 2	1	Pink colony	Negative rods	+	+	-	+	-	-	+	+	+	+	+	-	99.71%	99.11%	*Klebsiella pneumoniae*	37 (29.37%)
	2	Colorless colony	Negative rods	+	-	+	+	-	+	-	+	-	-	+	+	100.00%	99.62%	*Proteus vulgaris*	4 (3.18%)
	3	Pink colony	Negative rods	+	-	-	-	+	+	-	+	+	+	+	-	100.00%	99.76%	*Escherichia coli*	55 (43.65%)
	4	Colorless colony	Negative rods	+	-	+	+	-	-	+	+	-	+	+	-	100.00%	99.48%	*Enterobacter cloacae*	30 (23.80%)

MA: MacConkey agar; Rep.: Representative; Ca: Catalase; Ci: Citrate; Ox: Oxidase; Ur: Urease; In: Indole; Mr: Methyl red; Vp: Voges-Proskauer; G: Glucose; S: Sucrose; L: Lactose; Ga: Gas; Hs: Hydrogen sulphide; F: Fractional prevalence; Q: number of isolates examined; +: positive reaction; -: negative reaction.

Article DOI: 10.52589/AJENSR-YHDPSYTB
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Table 2: Taxonomic Characterization of Coliform Isolates in the Fluted Pumpkin Leaves from Farms

Sampled farms	Rep. isolates from leaves	Colonial and morphological characteristics		Biochemical characterizations												16S rRNA sequence homology		Identified coliforms	Frequency of occurrence of coliforms
		Growth on the MA Petri plates	Gram staining	Ca	Ox	Ur	Ci	In	Mr	Vp	Triple Sugar Iron test					16S similarity	16S identity		Farm 1 [F (%), Q = 90] Farm 2 [F (%), Q = 92]
											G	L	S	Ga	HS				
Farm 1	1	Colorless colony	Negative rods	+	-	-	+	-	+	-	+	-	-	+	+	100.00%	99.53%	*Salmonella enterica*	2 (2.22)
	2	Pink colony	Negative rods	+	-	-	-	+	+	-	+	+	+	+	-	100.00%	97.27%	*Escherichia coli*	30 (33.33)
	3	Pink colony	Negative rods	+	+	-	+	-	-	+	+	+	+	+	-	100.00%	98.75%	*Klebsiella pneumoniae*	23 (25.56)
	4	Colorless colony	Negative rods	+	-	+	+	-	+	-	+	-	-	+	+	100.00%	99.14%	*Proteus vulgaris*	3 (3.33)
	5	Colorless colony	Negative rods	+	-	+	+	-	-	+	+	-	+	+	-	100.00%	98.43%	*Enterobacter cloacae*	32 (35.56)
Farm 2	1	Pink colony	Negative rods	+	-	-	-	+	+	-	+	+	+	+	-	99.25%	97.77%	*Escherichia coli*	32 (34.78)
	2	Pink colony	Negative rods	+	+	-	+	-	-	+	+	+	+	+	-	100.00%	99.73%	*Klebsiella pneumoniae*	21 (22.83)
	3	Colorless colony	Negative rods	+	-	+	+	-	+	-	+	-	-	+	+	99.36%	98.41%	*Proteus vulgaris*	3 (3.26)
	4	Colorless colony	Negative rods	+	-	+	+	-	-	+	+	-	+	+	-	100.00%	99.21%	*Enterobacter cloacae*	36 (39.13)

MA: MacConkey agar; Rep.: Representative; Ca: Catalase; Ci: Citrate; Ox: Oxidase; Ur: Urease; In: Indole; Mr: Methyl red; Vp: Voges-Proskauer; G: Glucose; S: Sucrose; L: Lactose; Ga: Gas; Hs: Hydrogen sulphide; F: Fractional prevalence; Q: number of isolates examined; +: positive reaction; -: negative reaction.

Table 3: Taxonomic Characterization of Coliform Isolates in the Fluted Pumpkin Leaves from Open Markets

Sampled markets	Rep. isolates from markets	Colonial and morphological characteristics		Biochemical characterizations												16S rRNA sequence homology		Identified coliforms	Frequency of occurrence of coliforms
		Growth on the MA Petri plates	Gram staining	Ca	Ox	Ur	Ci	In	Mr	Vp	Triple Sugar Iron test					16S similarity	16S identity		F (%), Q = 100 F (%), Q = 107
											G	L	S	Ga	HS				
New-Benin market	1	Colorless colony	Negative rods	+	-	-	+	-	+	-	+	-	-	+	+	100.00%	99.36%	*Salmonella enterica*	5 (5.00)
	2	Pink colony	Negative rods	+	-	-	-	+	+	-	+	+	+	+	-	99.21%	96.75%	*Escherichia coli*	32 (32.00)
	3	Pink colony	Negative rods	+	+	-	+	-	-	+	+	+	+	+	-	98.17%	96.44%	*Klebsiella pneumoniae*	16 (16.00)
	4	Colorless colony	Negative rods	+	-	+	+	-	+	-	+	-	-	+	+	99.56%	98.46%	*Proteus vulgaris*	8 (8.00)
	5	Colorless colony	Negative rods	+	-	+	+	-	-	+	+	-	+	+	-	100.00%	98.48%	*Enterobacter cloacae*	35 (35.00)
	6	Pink colony	Negative rods	+	-	-	+	-	+	-	+	+	+	+	+	100.00%	97.58%	*Citrobacter freundii*	4(4.00)
Oba market	1	Pink colony	Negative rods	+	-	-	-	+	+	-	+	+	+	+	-	100.00%	99.17%	*Escherichia coli*	30 (28.04)
	2	Pink colony	Negative rods	+	+	-	+	-	-	+	+	+	+	+	-	96.38%	98.31%	*Klebsiella pneumoniae*	26 (24.30)
	3	Colorless colony	Negative rods	+	-	+	+	-	+	-	+	-	-	+	+	100.00%	99.48%	*Proteus vulgaris*	9 (8.41)
	4	Colorless colony	Negative rods	+	-	+	+	-	-	+	+	-	+	+	-	99.55%	97.58%	*Enterobacter cloacae*	39 (36.45)
	5	Pink colony	Negative rods	+	-	+	+	-	+	-	+	+	+	+	+	100.00%	98.42%	*Citrobacter freundii*	3 (2.80)

MA: MacConkey agar; Rep.: Representative; Ca: Catalase; Ci: Citrate; Ox: Oxidase; Ur: Urease; In: Indole; Mr: Methyl red; Vp: Voges-Proskauer; G: Glucose; S: Sucrose; L: Lactose; Ga: Gas; Hs: Hydrogen sulphide; F: Fractional prevalence; Q: number of isolates examined; +: positive reaction; -: negative reaction.

coli strain IM-OS11PF1, *K. pneumoniae* strain IM-OS12PF1, *P. vulgaris* strain IM-OS13PF1, and *E. cloacae* strain IM-OS14PF1 isolated from harvested fluted pumpkin leaves in Farm 1 have been deposited in the United States National Center for Biotechnological Information (NCBI) GenBank database under accession numbers PV012525, PV012526, PV012527,

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PV012528 and PV012529. Similarly, representative isolates of *E. coli* strain IM-OS15PF2, *K. pneumoniae* strain IM-OS16PF2, *P. vulgaris* strain IM-OS17PF2 and *E. cloacae* strain IM-OS18PF2 from the harvested pumpkin leaves in Farm 2 were deposited in the NCBI GenBank database under accession numbers PV012530, PV012531, PV012532 and PV012533.

The coliforms isolated from fluted pumpkin leaves sold in New-Benin and Oba Markets were generally similar to those found in harvested fluted pumpkin leaves at Farms 1 and 2 (Table 3). However, *Citrobacter freundii* which was not found in harvested fluted pumpkin leaves at the farms was isolated from the fluted pumpkin leaves sold in New-Benin and Oba Markets. *E. coli* was the most abundant coliform in fluted pumpkin leaves sold in New-Benin and Oba Markets while *C. freundii* was the least abundant coliform. Representative *S. enterica* strain IM-OS19PNM, *E. coli* strain IM-OS20PNM, *K. pneumoniae* strain IM-OS21PNM, *P. vulgaris* strain IM-OS22PNM, *E. cloacae* strain IM-OS23PNM and *C. freundii* strain IM-OS24PNM isolated from fluted pumpkin leaves sold in New-Benin Market have been deposited in the NCBI GenBank database under accession numbers PV012561, PV012562, PV012563, PV012564, PV012565 and PV012566. Similarly, representative isolates of *E. coli* strain IM-OS25PNM, *K. pneumoniae* strain IM-OS26PNM, *P. vulgaris* strain IM-OS27PNM, *E. cloacae* strain IM-OS28PNM and *C. frendii* strain IM-OS29PNM isolated from fluted pumpkin leaves sold in Oba Market were deposited in the NCBI GenBank database under Waccession numbers PV012567, PV012568, PV012569, PV012570 and PV012571.

Antibiotic Resistance Phenotype of the Coliforms

Antibiotic resistance profile of coliforms isolated from soil in Farms 1 and 2 is presented in Table 4. Generally, the prevalence of resistance of the coliforms from the soil in Farms 1 and 2 to the panel of tested antibiotics was highly variable. The coliforms were extremely highly resistant to amoxicillin and ceftriaxone antibiotics. Resistance to meropenem was not detected in coliforms isolated from soil in Farms 1 and 2. However, high to moderate levels of resistance to amoxicillin-clavulanic acid, ofloxacin, ciprofloxacin, gentamicin, sulfamethoxazole, tetracycline and chloramphenicol were detected.

Table 5 presents antibiotic resistance profile of coliforms isolated from harvested fluted pumpkin leaves at the farms. The antibiotic resistance pattern seen in coliforms from harvested fluted pumpkin leaves were similar to those observed in the farm soil.

Table 6 shows the antibiotic resistance profile of coliforms isolated from fluted pumpkin leaves sold at New-Benin and Oba markets. The antibiotic resistance pattern seen in coliforms from fluted pumpkin leaves were also similar to those observed in the harvested fluted pumpkin leaves at the farms.

Multiple Antibiotic Resistance Indices

Table 7 shows the multiple resistance indices of all coliforms in the soil from Farms 1 and 2. Of the 120 coliforms isolated from the soil of Farm 1, 56 coliforms were multidrug-resistant. Forty-eight (48) coliforms were multidrug-resistant out of the 126 coliforms isolated from Farm 2. MAR indices of 0.39 and 0.32 were reported for all 120 and 126 coliform isolates from soil of Farms 1 and 2, respectively.

Article DOI: 10.52589/AJENSR-YHDPSYTB
DOI URL: https://doi.org/10.52589/AJENSR-YHDPSYTB

African Journal of Environment and Natural Science Research
ISSN: 2689-9434
Volume 8, Issue 1, 2025 (pp. 236-257)
www.abjournals.org

The multiple resistance indices of all coliforms in harvested fluted pumpkin leaves from Farms 1 and 2 are presented in Table 8. Of the 90 coliforms isolated from the harvested fluted pumpkin leaves in Farm 1, 27 coliforms were multidrug-resistant. Thirty (30) coliforms were

Article DOI: 10.52589/AJENSR-YHDPSYTB
DOI URL: https://doi.org/10.52589/AJENSR-YHDPSYTB

African Journal of Environment and Natural Science Research
ISSN: 2689-9434
Volume 8, Issue 1, 2025 (pp. 236-257)

Table 4: Antibiotic Resistance Profile of Coliforms Isolated from the Farm Soil

Sampled farms	Identified coliforms	F	Antibiotic resistance prevalence of coliforms in farm soil									
			AX 30 µg N (%)	AMC 30 µg N (%)	OFX 5 µg N (%)	CIP 5 µg N (%)	CN 30 µg N (%)	CEF 10 µg N (%)	MEM 10 µg N (%)	SXT 25 µg N (%)	TET 15 µg N (%)	C 30 µg N (%)
Farm 1	*Escherichia coli*	52	48 (92.31)	26 (50.00)	22 (42.31)	25 (48.08)	21 (40.38)	47 (90.38)	0 (0.00)	21 (40.39)	29 (55.77)	16 (30.77)
	Salmonella enterica	2	2 (100.00)	1 (50.00)	0 (0.00)	0 (0.00)	0 (0.00)	2 (100.00)	0 (0.00)	1 (50.00)	1 (50.00)	1 (50.00)
	Klebsiella pneumoniae	40	38 (95.00)	22 (55.00)	20 (50.00)	21 (52.50)	19 (47.50)	37 (92.50)	0 (0.00)	20 (50.00)	25 (62.50)	15 (37.50)
	Proteus vulgaris	8	7 (87.50)	4 (50.00)	3 (37.50)	3 (37.50)	3 (37.50)	7 (87.50)	0 (0.00)	3 (37.50)	4 (50.00)	2 (25.00)
	Enterobacter cloacae	18	16 (88.89)	8 (44.44)	7 (38.89)	8 (44.44)	6 (33.33)	15 (83.33)	0 (0.00)	6 (33.33)	9 (50.00)	5 (27.78)
Farm 2	*Klebsiella pneumoniae*	37	34 (91.89)	19 (51.35)	12 (32.43)	12 (32.43)	13 (35.14)	30 (81.08)	0 (0.00)	17 (45.95)	22 (59.46)	12 (32.43)
	Proteus vulgaris	4	4 (100.00)	2 (50.00)	1 (25.00)	1 (25.00)	1 (25.00)	3 (75.00)	0 (0.00)	1 (25.00)	2 (50.00)	1 (25.00)
	Escherichia coli	55	49 (89.09)	23 (41.82)	21 (38.18)	22 (40.00)	19 (34.55)	47 (85.46)	0 (0.00)	20 (36.36)	29 (52.72)	14 (25.46)
	Enterobacter cloacae	30	25 (83.33)	12 (40.00)	11 (36.67)	12 (40.00)	14 (46.67)	24 (80.00)	0 (0.00)	9 (30.00)	14 (46.67)	8 (26.67)

F: number of coliform isolates; N: number of coliform isolates resistant to a specific antibiotic; CN: Gentamicin; MEM: Meropenem; AX: Amoxicillin; AMC: Amoxicillin-Clavulanic acid; OFX: Ofloxacin; CIP: Ciprofloxacin; SXT: Cotrimoxazole; CEF: Ceftriaxone; TET: Tetracycline; C: Chloramphenicol.

Article DOI: 10.52589/AJENSR-YHDPSYTB
DOI URL: https://doi.org/10.52589/AJENSR-YHDPSYTB

Table 5: Antibiotics Resistance Profile of Coliform Isolates in Fluted Pumpkin Leaves at Harvest in Farms

Sampled farms	Identified coliforms	F	Antibiotic resistance prevalence of coliforms in fluted pumpkin leaves from farms									
			AX 30 µg N (%)	AMC 30 µg N (%)	OFX 5 µg N (%)	CIP 5 µg N (%)	CN 30 µg N (%)	CEF 10 µg N (%)	MEM 10 µg N (%)	SXT 25 µg N (%)	TET 15 µg N (%)	C 30 µg N (%)
Farm 1	*Salmonella enterica*	2	2 (100.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (50.00)	0 (0.00)	1 (50.00)	1 (50.00)	0 (0.00)
	Escherichia coli	30	27 (90.00)	12 (40.00)	9 (30.00)	11 (36.67)	8 (26.67)	24 (80.00)	0 (0.00)	9 (30.00)	12 (40.00)	3 (10.00)
	Klebsiella pneumoniae	23	21 (91.30)	9 (39.13)	8 (34.78)	9 (39.13)	6 (26.09)	19 (82.61)	0 (0.00)	9 (39.13)	11 (47.83)	5 (21.74)
	Proteus vulgaris	3	2 (66.67)	1 (33.33)	1 (33.33)	1 (33.33)	1 (33.33)	2 (66.67)	0 (0.00)	1 (33.33)	1 (33.33)	1 (33.33)
	Enterobacter cloacae	32	26 (81.25)	10 (31.25)	8 (25.00)	10 (31.25)	7 (21.88)	25 (78.13)	0 (0.00)	7 (21.88)	12 (37.50)	5 (15.63)
Farm 2	*Escherichia coli*	32	27 (84.38)	10 (31.25)	26 (81.25)	11 (34.38)	10 (31.25)	25 (78.13)	0 (0.00)	9 (28.13)	14 (43.75)	6 (18.75)
	Klebsiella pneumoniae	21	17 (80.95)	8 (38.10)	6 (28.57)	5 (23.81)	6 (28.57)	15 (71.43)	0 (0.00)	8 (38.10)	11 (52.38)	5 (23.81)
	Proteus vulgaris	3	2 (66.67)	1 (33.33)	1 (33.33)	1 (33.33)	1 (33.33)	1 (33.33)	0 (0.00)	1 (33.33)	1 (33.33)	1 (33.33)
	Enterobacter cloacae	36	29 (80.56)	11 (30.56)	11 (30.56)	11 (30.56)	13 (36.11)	27 (75.00)	0 (0.00)	9 (25.00)	14 (38.89)	8 (22.22)

F: number of coliform isolates; N: number of coliform isolates resistant to a specific antibiotic; CN: Gentamicin; MEM: Meropenem; AX: Amoxicillin; AMC: Amoxicillin-Clavulanic acid; OFX: Ofloxacin; CIP: Ciprofloxacin; SXT: Cotrimoxazole; CEF: Ceftriaxone; TET: Tetracycline; C: Chloramphenicol.

Table 6: Antibiotics Resistance Profile of Coliform Isolates in Fluted Pumpkin Leaves Sold at the Open Markets

Sampled markets	Identified coliforms	F	Antibiotics resistance prevalence of coliforms in fluted pumpkin leaves at the open markets									
			AX 30 µg N (%)	AMC 30 µg N (%)	OFX 5 µg N (%)	CIP 5 µg N (%)	CN 30 µg N (%)	CEF 10 µg N (%)	MEM 10 µg N (%)	SXT 25 µg N (%)	TET 15 µg N (%)	C 30 µg N (%)
New-Benin market	*Salmonella enterica*	5	4 (80.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	2 (40.00)	0 (0.00)	2 (40.00)	2 (40.00)	0 (0.00)
	Escherichia coli	32	27 (84.38)	11 (34.38)	7 (21.88)	9 (28.13)	6 (18.75)	21 (65.63)	0 (0.00)	7 (21.88)	10 (31.25)	2 (6.25)
	Klebsiella pneumoniae	16	13 (81.25)	5 (31.25)	4 (25.00)	5 (31.25)	3 (18.75)	11 (68.75)	0 (0.00)	5 (31.25)	6 (37.50)	2 (12.50)
	Proteus vulgaris	8	4 (50.00)	2 (25.00)	2 (25.00)	2 (25.00)	2 (25.00)	4 (50.00)	0 (0.00)	2 (25.00)	2 (25.00)	2 (25.00)
	Enterobacter cloacae	35	26 (74.29)	9 (25.71)	6 (17.14)	8 (22.86)	6 (17.14)	24 (68.57)	0 (0.00)	6 (17.14)	10 (28.57)	3 (8.57)
	Citrobacter freundii	4	2 (50.00)	1 (25.00)	1 (25.00)	1 (25.00)	1 (25.00)	1 (25.00)	0 (0.00)	1 (5.00)	1 (25.00)	0 (0.00)
Oba market	*Escherichia coli*	30	24 (80.00)	11 (36.67)	8 (26.67)	9 (30.00)	7 (23.33)	18 (60.00)	0 (0.00)	8 (26.67)	7 (23.33)	3 (10.00)
	Klebsiella pneumoniae	26	22 (84.62)	9 (34.62)	8 (30.77)	9 (34.62)	6 (23.08)	16 (61.54)	0 (0.00)	7 (26.92)	8 (30.77)	4 (15.39)
	Proteus vulgaris	9	5 (55.56)	3 (33.33)	3 (33.33)	2 (22.22)	3 (33.33)	4 (44.44)	0 (0.00)	2 (22.22)	2 (22.22)	2 (22.22)
	Enterobacter cloacae	39	31 (79.49)	14 (35.90)	12 (30.77)	13 (33.33)	11 (28.21)	24 (61.54)	0 (0.00)	9 (23.08)	10 (25.64)	7 (17.95)
	Citrobacter freundii	3	2 (66.67)	1 (33.33)	1 (33.33)	1 (33.33)	1 (33.33)	1 (33.33)	0 (0.00)	1 (33.33)	1 (33.33)	0 (0.00)

F: number of coliform isolates; N: number of coliform isolates resistant to a specific antibiotic; CN: Gentamicin; MEM: Meropenem; AX: Amoxicillin; AMC: Amoxicillin-Clavulanic acid;

Article DOI: 10.52589/AJENSR-YHDPSYTB
DOI URL: https://doi.org/10.52589/AJENSR-YHDPSYTB

African Journal of Environment and Natural Science Research
ISSN: 2689-9434
Volume 8, Issue 1, 2025 (pp. 236-257)
www.abjournals.org

OFX: Ofloxacin; CIP: Ciprofloxacin; SXT: Cotrimoxazole; CEF: Ceftriaxone; TET: Tetracycline; C: Chloramphenicol.

Table 7: Multiple Antibiotics Resistance Indices in Coliforms from Farm Soil

Sampled soil	Identified coliforms	Coliforms			
		A	∑*AR*	*B*	MAR indices
Soil from farm 1	*Escherichia coli*	7	144	52	0.40
	Salmonella enterica	7	6	2	0.43
	Klebsiella pneumoniae	7	121	40	0.43
	Proteus vulgaris	7	19	8	0.34
	Enterobacter cloacae	7	41	18	0.33
	All isolates	7	331	120	0.39
Soil from farm 2	*Klebsiella pneumoniae*	7	95	37	0.37
	Proteus vulgaris	7	8	4	0.29
	Escherichia coli	7	113	49	0.33
	Enterobacter cloacae	7	67	36	0.27
	All isolates	7	283	126	0.32

MAR: Mean multiple antibiotic resistance indices. ∑AR: Sum of antibiotic resistance scores of each coliform isolate, defined as the sum of antibiotic classes to which a particular coliform isolates exhibited resistance. *A*: Total number of antibiotic classes tested. *B*: total count of coliform isolates examined.

Table 8: Multiple Antibiotics Resistance Indices in Coliforms from Fluted Pumpkin Leaves at Harvest in Farms

Sampled pumpkin leaves from farms	Identified coliforms	Coliforms			
		A	∑*AR*	*B*	MAR indices
Pumkin leaves from farm 1	*Salmonella enterica*	7	2	2	0.14
	Escherichia coli	7	53	30	0.25
	Klebsiella pneumoniae	7	47	23	0.29
	Proteus vulgaris	7	6	3	0.29
	Enterobacter cloacae	7	49	32	0.22
	All coliform isolates	7	157	90	0.25
Pumpkin leaves from farm 2	*Escherichia coli*	7	62	32	0.28
	Klebsiella pneumoniae	7	43	21	0.29
	Proteus vulgaris	7	6	3	0.29
	Enterobacter cloacae	7	62	36	0.25
	All coliform isolates	7	173	92	0.27

MAR: Mean multiple antibiotic resistance indices. ∑AR: Sum of antibiotic resistance scores of each coliform isolate, defined as the sum of antibiotic classes to which a particular coliform

Article DOI: 10.52589/AJENSR-YHDPSYTB
DOI URL: https://doi.org/10.52589/AJENSR-YHDPSYTB

isolates exhibited resistance. *A*: Total number of antibiotic classes tested. *B*: total count of coliform isolates examined multidrug-resistant out of the 92 coliforms isolated from the harvested fluted pumpkin leaves in Farm 2. MAR indices of 0.25 and 0.27 were reported for all 90 and 92 coliform isolates from the harvested fluted pumpkin leaves in Farms 1 and 2, respectively.

The multiple resistance indices of all coliforms in fluted pumpkin leaves sold at New-Benin and Oba Markets are shown in Table 9. Of the 100 coliforms isolated from the fluted pumpkin leaves sold in New-Benin Market, 22 coliforms were multidrug-resistant. Twenty-seven (27) coliforms were multidrug-resistant out of the 107 coliforms isolated from fluted pumpkin leaves sold at Oba Market. MAR indices of 0.18 and 0.20 were reported for all 100 and 107 coliform isolates from the fluted pumpkin leaves sold at New-Benin and Oba Markets, respectively.

Prevalence of Coliforms

Table 10 shows the prevalence of coliform isolates in the farm soil. Of a total of 150 presumptive coliform isolates examined in Farm 1 from Day 0 to Day 70 of the research, 120 isolates were confirmed as coliforms. Out of the 120 confirmed coliform isolates, 56 coliforms were found as multidrug-resistant coliforms. Of a total of 150 presumptive coliform isolates examined in Farm 2 from Day 0 to Day 70 of the research, 126 isolates were confirmed as coliforms. Out of the 126 confirmed coliform isolates, 48 coliforms were multidrug-resistant.

The prevalence of coliform isolates in the harvested fluted pumpkin leaves at the farms is presented in Table 11. Of a total of 120 presumptive coliform isolates examined in Farm 1 from Day 42 to Day 70 of the research, 90 isolates were confirmed as coliforms. Out of the 90 confirmed coliform isolates, 27 coliforms were multidrug-resistant coliforms. Of a total of 120 presumptive coliform isolates examined in Farm 2 from Day 42 to Day 70 of the research, 92 isolates were confirmed as coliforms. Out of the 92 confirmed coliform isolates, 32 coliforms were multidrug-resistant coliforms.

Table 9: Multiple Antibiotics Resistance Indices in Coliforms from Fluted Pumpkin Leaves Sold at the Open Markets

Sampled pumpkin leaves from markets	Identified coliforms	Coliforms			
		A	∑*AR*	*B*	MAR indices
Pumkin leaves from New-Benin market	*Salmonella enterica*	7	4	5	0.11
	Escherichia coli	7	43	32	0.19
	Klebsiella pneumoniae	7	25	16	0.22
	Proteus vulgaris	7	12	8	0.21
	Enterobacter cloacae	7	40	35	0.16
	Citrobacter freundii	7	5	4	0.18
	All coliform isolates	7	129	100	0.18
Pumpkin leaves from Oba market	*Escherichia coli*	7	44	30	0.21
	Klebsiella pneumoniae	7	42	26	0.23
	Proteus vulgaris	7	14	9	0.22
	Enterobacter cloacae	7	46	39	0.71
	Citrobacter freundii	7	5	3	0.24
	All coliform isolates	7	151	107	0.20

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DOI URL: https://doi.org/10.52589/AJENSR-YHDPSYTB

MAR: Mean multiple antibiotic resistance indices. ∑AR: Sum of antibiotic resistance scores of each coliform isolate, defined as the sum of antibiotic classes to which a particular coliform isolates exhibited resistance. *A*: Total number of antibiotic classes tested. *B*: total count of coliform isolates examined.

Table 10: Prevalence of Coliform Isolates in Farm Soil

Sampled farms	Days of sample collection	Prevalence estimates	
		t (*H*)	u (*K*)
Farm 1	Day 0, g = 30	22 (0.73)	8 (0.36)
	Day 42, g = 40	32 (0.80)	17 (0.53)
	Day 56, g = 40	30 (0.75)	12 (0.40)
	Day 70, g = 40	36 (0.90)	19 (0.52)
Farm 2	Day 0, g = 30	24 (0.80)	6 (0.25)
	Day 42, g = 40	34 (0.85)	16 (0.47)
	Day 56, g = 40	33 (0.83)	12 (0.36)
	Day 70, g = 40	35 (0.88)	14 (0.40)

g: number of presumptive coliform isolates examined; t: number of confirmed coliforms; u: number of multidrug-resistant coliforms; *H*: fractional prevalence of presumptive coliform isolates; *K*: fractional prevalence of multidrug-resistant coliforms.

Table 11: Prevalence of Coliform Isolates in Fluted Pumpkin Leaves at Harvest in Farms

Sampled pumpkin leaves	Days of sample collection	Prevalence estimates	
		t (*H*)	u (*K*)
Farm 1	Day 42, g = 40	26 (0.65)	7 (0.27)
	Day 56, g = 40	36 (0.90)	9 (0.25)
	Day 70, g = 40	28 (0.70)	11 (0.39)
Farm 2	Day 42, g = 40	28 (0.70)	9 (0.32)
	Day 56, g = 40	34 (0.85)	12 (0.35)
	Day 70, g = 40	30 (0.75)	11 (0.37)

g: number of presumptive coliform isolates examined; t: number of confirmed coliforms; u: number of multidrug-resistant coliforms; *H*: fractional prevalence of presumptive coliform isolates; *K*: fractional prevalence of multidrug-resistant coliforms.

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African Journal of Environment and Natural Science Research
ISSN: 2689-9434
Volume 8, Issue 1, 2025 (pp. 236-257)

The prevalence of coliform isolates in the fluted pumpkin leaves sold at New-Benin and Oba Markets is presented in Table 12. Of a total of 120 presumptive coliform isolates examined in fluted pumpkin leaves sold at New-Benin Market during Day 42 to Day 70 of the research, 100 isolates were confirmed as coliforms. Out of the 100 confirmed coliform isolates, 21 coliforms were found to be multidrug-resistant coliforms. Of a total of 120 presumptive coliform isolates examined in fluted pumpkin leaves sold at Oba Market during Day 42 to Day 70 of the research, 107 isolates were confirmed as coliforms. Out of the 107 confirmed coliform isolates, 27 coliforms were multidrug-resistant coliforms.

Counts of Coliforms

The mean coliform counts in soil from the farms is presented in Table 13. Confirmed total coliform counts in the soil of Farm 1 during Day 0 and Day 70 of the research ranged from 3.12 ± 2.01 $\log_{10}$ CFU/g to 4.47 ± 3.11 $\log_{10}$ CFU/g, and from 3.29 ± 2.10 $\log_{10}$ CFU/g to 4.51 ± 3.15 $\log_{10}$ CFU/g in Farm 2. In Farm 1, the counts of multidrug-resistant coliforms were between 2.68 ± 1.57 $\log_{10}$ CFU/g and 4.18 ± 2.82 $\log_{10}$ CFU/g. The counts of multidrug-resistant coliforms in the soil of Farm 2 ranged from 2.69 ± 1.51 $\log_{10}$ CFU/g to 4.11 ± 2.75 $\log_{10}$ CFU/g. There was no significant difference ($p > 0.05$) between the counts of multidrug-resistant coliforms in soil from Farms 1 and 2.

The mean coliform counts in harvested fluted pumpkin leaves from the farms is shown in Table 14. Confirmed total coliform counts in harvested fluted pumpkin leaves of Farm 1 during Day 56 to Day 70 of the research ranged from 2.23 ± 0.63 $\log_{10}$ CFU/g to 2.34 ± 0.60 $\log_{10}$ CFU/g, and from 2.20 ± 0.82 $\log_{10}$ CFU/g to 2.34 ± 0.64 $\log_{10}$ CFU/g in harvested fluted pumpkin leaves of Farm 2. In Farm 1, the counts of multidrug-resistant coliforms in harvested fluted pumpkin leaves were between 1.72 ± 0.79 $\log_{10}$ CFU/g and 1.82 ± 0.78 $\log_{10}$ CFU/g.

Table 12: Prevalence of Coliform Isolates in Fluted Pumpkin Leaves Sold at the Open Markets

Sampled pumpkin leaves	Days of sample collection	Prevalence estimates	
		t (*H*)	u (*K*)
New-Benin market	Day 42, g = 40	36 (0.90)	8 (0.22)
	Day 56, g = 40	30 (0.75)	6 (0.20)
	Day 70, g = 40	34 (0.85)	7(0.21)
Oba market	Day 42, g = 40	33 (0.83)	8 (0.24)
	Day 56, g = 40	36 (0.90)	10 (0.28)
	Day 70, g = 40	38 (0.95)	9 (0.24)

g: number of presumptive coliform isolates examined; t: number of confirmed coliforms; u: number of multidrug-resistant coliforms; *H*: fractional prevalence of presumptive coliform isolates; *K*: fractional prevalence of multidrug-resistant coliforms.

Article DOI: 10.52589/AJENSR-YHDPSYTB
DOI URL: https://doi.org/10.52589/AJENSR-YHDPSYTB

Table 13: Coliform Counts in Farm Soil

Sampled farms	Days of sample collection	Mean Coliform counts Log_{10} CFU/g		
		Presumptive total coliform count (*pTCC*) E = 3	Confirmed total coliform count (c*TCC*) E = 3	Multidrug-resistant coliform count (m*TCC*) E = 3
Farm 1	Day 0	3.25 ± 2.15	3.12 ± 2.01	2.68 ± 1.57
	Day 42	4.40 ± 3.15	4.31 ± 3.05	4.03 ± 2.78
	Day 56	4.47 ± 3.37	4.35 ± 3.25	3.95 ± 2.85
	Day 70	4.51 ± 3.16	4.47 ± 3.11	4.18 ± 2.82
Farm 2	Day 0	3.39 ± 2.05	3.29 ± 2.10	2.69 ± 1.51
	Day 42	4.52 ± 3.23	4.45 ± 3.01	4.12 ± 2.68
	Day 56	4.57 ± 3.15	4.49 ± 3.22	4.05 ± 2.78
	Day 70	4.56 ± 3.01	4.51 ± 3.15	4.11 ± 2.75

E: Triplicate composite samples

Table 14: Coliform Counts in Fluted Pumpkin Leaves at Harvest in Farms

Sampled pumpkin leaves from farms	Days of sample collection	Coliform counts Log_{10} CFU/g		
		Presumptive total coliform count (*pTCC*)	Confirmed total coliform count (c*TCC*)	Multidrug-resistant coliform count (m*TCC*)
Farm 1	Day 42	2.52 ± 0.64	2.34 ± 0.60	1.77 ± 0.82
	Day 56	2.45 ± 0.62	2.32 ± 0.61	1.72 ± 0.79
	Day 70	2.38 ± 0.63	2.23 ± 0.63	1.82 ± 0.78
Farm 2	Day 42	2.36 ± 0.61	2.20 ± 0.82	1.71 ± 0.73
	Day 56	2.42 ± 0.63	2.34 ± 0.64	1.89 ± 0.55
	Day 70	2.40 ± 0.62	2.27 ± 0.79	1.84 ± 0.52

Triplicate composited soil samples were collected from the farms

The counts of multidrug-resistant coliforms in harvested fluted pumpkin leaves of Farm 2 ranged from 1.71 ± 0.73 log_{10} CFU/g to 1.89 ± 0.55 log_{10} CFU/g. There was no significant

Article DOI: 10.52589/AJENSR-YHDPSYTB
DOI URL: https://doi.org/10.52589/AJENSR-YHDPSYTB

difference ($p > 0.05$) between the counts of multidrug-resistant coliforms in harvested fluted pumpkin leaves from Farms 1 and 2.

The mean coliform counts in fluted pumpkin leaves sold at New-Benin and Oba Markets are shown in Table 15. Confirmed total coliform counts in fluted pumpkin leaves sold at New-Benin Market during Day 56 to Day 70 of the research ranged from 2.15 ± 0.67 $\log_{10}$ CFU/g to 2.34 ± 0.73 $\log_{10}$ CFU/g, and from 2.22 ± 0.66 $\log_{10}$ CFU/g to 2.29 ± 0.65 $\log_{10}$ CFU/g in fluted vegetables sold at Oba Market. In the fluted pumpkin leaves sold at New-Benin Market, the counts of multidrug-resistant coliforms were between 1.26 ± 0.76 $\log_{10}$ CFU/g to 1.68 ± 0.73 $\log_{10}$ CFU/g. The counts of multidrug-resistant coliforms in fluted pumpkin leaves sold at Oba Market ranged from 1.60 ± 0.76 $\log_{10}$ CFU/g to 1.74 ± 0.72 $\log_{10}$ CFU/g. There was no significant difference ($p > 0.05$) between the counts of multidrug-resistant coliforms in fluted pumpkin leaves sold at New-Benin Market and those in fluted pumpkin leaves sold at Oba Market.

Table 15: Coliform Counts in Fluted Pumpkin Leaves Sold at the Open Markets

Sampled markets	Days of sample collection	Coliform counts $\log_{10}$ CFU/g		
		Presumptive total coliform count (*pTCC*)	Confirmed total coliform count (c*TCC*)	Multidrug-resistant coliform count (m*TCC*)
New-Benin market	Day 42	2.39 ± 0.82	2.34 ± 0.73	1.68 ± 0.73
	Day 56	2.27 ± 0.62	2.15 ± 0.67	1.45 ± 0.71
	Day 70	2.25 ± 0.64	2.18 ± 0.69	1.26 ± 0.76
Oba market	Day 42	2.30 ± 0.66	2.22 ± 0.66	1.60 ± 0.76
	Day 56	2.37 ± 0.71	2.29 ± 0.65	1.74 ± 0.72
	Day 70	2.30 ± 0.73	2.27 ± 0.64	1.65 ± 0.74

Triplicate composited soil samples were collected from the farms

DISCUSSION

Fluted pumpkin leaves and other veggies are really vulnerable to contamination by microorganisms from soil, water, and handling during harvest or afterwards. This means they can carry a variety of bacteria, including pathogens that affect both humans and plants. In our research, we found coliforms like *K. pneumoniae*, *E. coli*, *P. vulgaris*, and *E. cloacae* in all the fluted pumpkin leaves and soil samples we examined, while *C. freundi* was only detected in samples from open markets (Tables 1 to 3). Similar studies show that *E. coli* is often found in various vegetables [15, 16, 17]. *E. coli* was the most common coliform in our fluted pumpkin and soil samples (Tables 1 to 3). The prevalence of coliforms here could be due to contaminated

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soil, poor storage, and farming practices. The structure of fluted pumpkin leaves, with their folds and high surface area, might also attract these microorganisms [16, 17].

We discovered that many of the coliforms were resistant to antibiotics like amoxicillin and ceftriaxone, and showed moderate resistance to ofloxacin, ciprofloxacin, gentamicin, and others (Tables 4 to 6). This likely stems from the widespread, unregulated use of these antibiotics in Nigeria. We saw more resistant coliforms in the soil than in the pumpkin leaves (Tables 4 to 6). Interestingly, we did not find any resistance to meropenem, which is consistent with similar previous studies [15, 18]. This finding also agreed with the carbapenem activity reported in previous studies of Ramadan *et al*. [19] and Igbinosa *et al*. [16]. Eating contaminated food is just one-way humans come into contact with antibiotic-resistant bacteria [20]. The risks from various exposure routes are mostly unknown but deserve attention in health risk assessments. Some researchers think that antibiotic resistance through vegetables is a big enough concern, so that people should wash and peel produce carefully [21].

We also found multidrug-resistant coliforms in both pumpkin leaves and farm soil (Tables 7 to 9). The highest resistance indices were in the soil, while the lowest were in leaves from New-Benin and Oba Markets. The indices in soil samples suggested that manure-treated soils could be a source for multidrug-resistant coliforms, while those in the leaves from the markets indicated they might not be a major risk. This could be due to cleaning practices like washing leaves by farmers and retailers. Studies suggest that soil-grown veggies might have a higher risk of encountering antibiotic-resistant bacteria compared to those grown above ground [22, 23]. So, different growing conditions and farming techniques can lead to varying patterns of antibiotic resistance.

The coliform counts found in this study (Tables 13 to 15) matched those reported by Hassan et al. [24], Schwaiger et al. [25] and Marti et al. [15]. Also, they aligned with specific coliform counts from Lee et al. [17] and Igbinosa et al. [16]. However, comparing these studies is tough because differences in laboratory methods, sampling, and handling can skew results. Still, using selective media with antibiotics or screening for bacteria has consistently shown a high presence of antibiotic-resistant bacteria on fresh produce.

Coliform contamination may stem from sources like fecal contamination, poor water quality, and inadequate handling practices. To reduce the risk of antibiotic resistance from fresh vegetables, it is essential to ensure cleanliness in growing practices that also limit exposure to antibiotic-resistant bacteria found in animal and human digestion. It is important to investigate how waste treatment methods (like composting and lime stabilization) can effectively eliminate antibiotic resistance from manure and human waste. We also need to verify the safety periods between applying fecal materials and harvesting crops to guarantee that crops remain safe and free from antibiotic resistance, especially under different climate conditions.

CONCLUSION

This research shows that various coliforms such as *K. pneumoniae*, *E. coli*, *P. vulgaris*, *E. cloacae*, and *C. freundii* were found in fluted pumpkin leaves grown in manure-amended soil in Benin City, Edo State, Nigeria. These coliforms showed high resistance to amoxicillin and ceftriaxone, and moderate resistance to other antibiotics like ofloxacin and ciprofloxacin, but

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no resistance to meropenem. Multidrug resistance was noted, especially linked to manure-amended soils, which are risky sources of multidrug-resistant coliforms. On the other hand, fluted pumpkin leaves themselves were not seen as a major risk. It is recommended that good agricultural practices be followed early on to prevent spreading antibiotic-resistant bacteria in the environment and to human consumers. Food processors and consumers should ensure thorough cleaning of fluted pumpkin leaves or other produce before use or consumption.

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Article DOI: 10.52589/AJENSR-YHDPSYTB
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