***Original Research Article***

# CHARACTERIZATION AND ANTIMICROBIAL RESISTANCE PROFILE OF PATHOGENIC BACTERIA ISOLATED FROM FRESHLY SOLD *AMARANTHUS VIRIDIS* IN ILE-IFE, SOUTHWEST NIGERIA.


## ABSTRACT

*Amaranthus viridis* is known to have excellent nutritional value because of its high content of essential micronutrients which are considered heat labile, thus little or no heat is applied during its preparation to destroy microbial contaminants acquired during planting, harvesting or processing. This study was conducted to characterize pathogenic bacteria isolated from freshly sold *Amaranthus viridis* and determine their susceptibilities to commonly used antibiotics. Fresh, green and firm *Amaranthus viridis* were collected at different retail and cultivation sites across Ife Central Local Government Area of Ile–Ife and microbiologically assayed for the presence of pathogenic bacteria such as *Shigella* species and *Escherichia coli* using standard methods described by APHA. The result shows that 21 isolates were recovered of which 7(23.33%) showed characteristics of *Shigella* which appear colourless without a black centre on SSA and 5(16.7%) were typical of *Escherichia coli* with characteristic green metallic sheen on EMB agar. The isolates were 100% sensitive to ofloxacilin, more than 86% of the isolated *Shigella* spp. and *Escherichia coli* exhibited multi resistance to other antibiotics especially nitrofurantoin and amoxicillin. This study concludes that the freshly sold *Amaranthus viridis* in Ile-Ife were contaminated with pathogenic bacteria, hence, the result creates awareness on the dangers of consuming these vegetables.



**Keywords**: *Amaranthus viridis*, Antibiotic Resistance, Enteropathogens, *Escherichia coli*, *Shigella* spp.

## 1. INTRODUCTION

Vegetables are known to be extraordinary dietary source rich in vitamins, iron, calcium, proteins, fats, minerals, dietary fibres and other nutrients like flavonoids, carotenoids and phenolic compounds that may lower the risk of cancer, heart disease and other illnesses [1]. *Amaranthus viridis* also known as inine ogwu (igbo), efo tete(Yoruba), namijin gaasayaa (hausa) is a leafy vegetable which belongs to the family *Amaranthaceae* used as fodder and in medicine. It possesses slender inflorescences spikes, not spiky, trimers female flowers, strongly verrucose, apiculate, as long as the perianth, slightly compressed, margin acute and glossy black [2].

In Africa, Amaranths are among the most important leafy vegetables, a fact attributed to their ease of cultivation, wide occurrence, low pests and diseases incidence, low labour input, ease in cooking and high nutritional value. Despite its ample health benefits, consumption of vegetables has been implicated as a potential vehicle for the transmission of bacterial, parasitic and viral pathogens implicated in enteric fever. According to Centers for Disease Prevention and Control (CDC), an estimated annual incidence of 22 million cases of enteric fever occur resulting in 200,000 deaths worldwide [3]. [4,5] reported more than 90 percent food poisoning cases attributable to enteropathogens including; *Salmonella*, *Shigella*, *Clostridium perfringes*, *Escherichia coli*, *Proteus* each year.

*Shigella* spp are small Gram negative bacteria of the *Enterobacteriaceae* family, the causative agents of shigellosis, also known as bacillary dysentery. Once ingested, *Shigella* spp survive the acidic environment of the stomach and invade the epithelial cells of the colon to initiate infection [6]. Aside the virulene genes contained in their chromosomes, *Shigella* spp possess virulence plasmids that encode genes involved in the invasion process and intra- and inter-cellular spread [7]. *Escherichia coli* on the other hand is a motile, non-spore forming facultative anaerobe that colonizes the human gut. Most strains are harmless and constitute part of the normal intestinal microflora. These strains serve a useful function in the body by suppressing the growth of harmful bacteria and by synthesizing appreciable amounts of vitamins. However, based on unique virulence factors, six pathogenic groups have been identified; enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC) , enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) [8,9]. Of these, only the first four (4)

groups have been implicated in food or water borne disease. Microbiological contamination of fruit and vegetables can occur directly or indirectly from animals or insects, soil, manures, equipment used in growing, as well as human handling along the food chain. The continued use of untreated waste water and manure in developing countries as fertilizers for the production of raw vegetables are major contributing causes of numerous food borne disease outbreaks. Raw vegetables harbour a number of pathogenic microorganisms including *Salmonella*, *Escherichia coli*, *Klebsiella* species, *Mycobacterium* species and *Listeria monocytogenes* obtained from manures used to promote the growth of these vegetables, this poses a great risk to public health. In addition, abuse of antibiotics and absence of basic sanitation facilities (e.g. toilets) particularly in rural Sub-Saharan Africa are factors that have contributed to the development of antibiotic resistant bacteria which find their way through sewerages into agricultural farms [10]. *Amaranth viridis* is an essential component of our diets but may also harbour pathogenic microorganisms in its unprepared or poorly prepared state which may result in an array of food borne diseases. Hence, this study was designed to characterize the probable pathogenic bacteria isolated from freshly sold *Amaranth* v*iridis* in Ile-Ife and the resistance to commercially sold antibiotics.

## 2. MATERIALS AND METHODS

### 2.1 Study area

The study area, Ile-Ife is an ancient town in South Western Nigeria about 218 kilometers Northeast of Lagos with a population of about 755,260 persons. Ile-Ife covers a total land mass of 1,791km$^2$. Geographically, the study area lies within latitudes 7$^0$28'N and 7$^0$46'N, and longitudes 4$^0$36'E and 4$^0$56' E (figure 1).

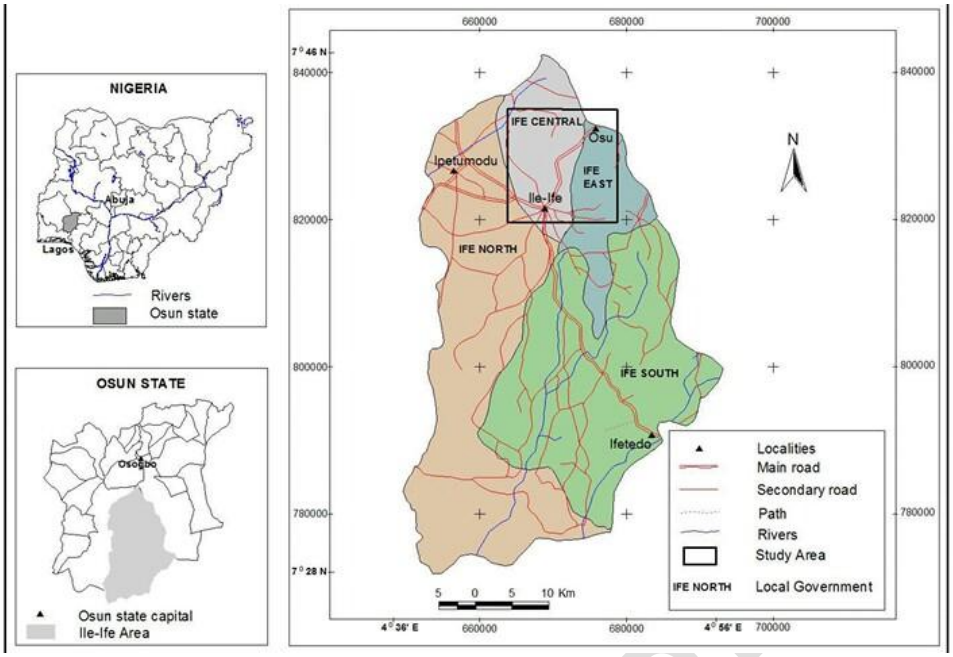


**Fig. 1**: Location of the study area, Ile-Ife, Osun State, Nigeria

(Source: Digital archives of the Department of Geography, Obafemi Awolowo University, Ife)

## 2.2 Sample collection

Samples were collected from vegetables sellers in markets and in small farms in Ife Central Local Government Area, Osun-State. A total of 30 samples of fresh green vegetable with approximately five stalks were collected in sterile Ziploc bags and brought to the Microbiology laboratory of Obafemi Awolowo University for bacteriological analysis.

## 2.3 Preparation of Media

All media used were prepared according to manufacturer's instruction and sterilized in an autoclave at 121$^{o}$C for 15 minutes (except for Selenite broth and *Salmonella-Shigella* agar which do not require sterilization).

## 2.4 Bacteriological analyses

All samples were processed in accordance with the standard methods of the American Public Health Association [11]. Approximately five (5) stalks of each vegetable sample were dropped each into one Ziploc bag and sterile distilled water (10ml) was used to wash the samples in the Ziploc bags thoroughly.

### 2.4.1 Isolation of *Shigella* spp

Exactly 2 ml each of the wash water was dispensed into 10 ml of Selenite broth for enrichment and incubated at 37$^{o}$C for 24 hours. A loopful of enriched samples in the Selenite broth was then streaked on already prepared SSA plates. The plates were incubated at 37$^{o}$C for 24 hours. After 24 hours, the plates were examined for colourless colonies without black centres on SSA plates. Also, 2ml each of the rinse water was enriched in 10 ml of Nutrient broth and incubated at 37$^{o}$C for 24 hours. A loopful of enriched samples in the Nutrient broth was then streaked on already prepared SSA plates, inverted and incubated at 37$^{o}$C for 24 hours. After 24 hours, the plates were examined for colourless colonies without black centres on SSA plates. Lastly, a loopful of the rinse water was streaked on already prepared SSA plates and incubated at 37$^{o}$C for 24 hours [11].

### 2.4.2 Isolation of *Escherichia coli*

*E. coli* was isolated using the method of APHA as described for *Shigella* spp above but the culturing was done on prepared EMB plates against SSA for *Shigella* spp. After 24 hours, the plates were examined for colonies with green metallic sheen appearance.

### 2.4.3 Purification of isolates

Sub culturing was done on solidified sterile nutrient agar to obtain pure cultures. The pure cultures were maintained at 4°C in nutrient agar as stock culture for further tests [12].

### 2.4.4 Characterization and Identification of isolates

Isolates were characterized and identified using biochemical procedures (Gram's reaction,catalase, oxidase, citrate utilization, urease, methyl red-voges proskaeur test, indole, hydrogen sulphide test, motility test, lactose fermentation, sucrose fermentation and glucose fermentation) according to protocols described in Bergey's manual of Systemic Bacteriology [13].

### 2.4.5 Determination of Antibiotic sensitivity

Agar disc diffusion was used for Antibiotics sensitivity testing according to the method of [14]. A 24 hour old culture was inoculated into a10ml sterile distilled water in a test tube to give a concentration of one million colony forming units per ml and standardized to a turbidity of 0.5 MacFarland. Antibiotic impregnated Gram-negative single discs containing; Tetracycline (30µg), Ceftriaxone (30µg), Gentamicin (10µg), Amoxicillin (30µg), Ofloxacin (5µg), Augmentin (30µg), Nitrofurantoin (300µg) and Ciprofloxacin (5µg) were aseptically placed on inoculated agar using sterile forceps and incubated at $37^0$C for 18-24 hours. The zone of inhibition was recorded in mm and interpreted according to Clinical Laboratory Standard [15].

#### *2.4.5.1 Multiple Antibiotic Resistance (MAR) index of the isolates*

The Multiple Antibiotic Resistance (MAR) index was determined as the ratio of the number of antibiotics to which an isolate showed resistance to the total number of antibiotics tested.

## 3. RESULTS

### 3.1 Microbial load of the samples collected

Based on colony morphology, Gram's reaction and biochemical tests carried out, a total of twenty-one (21) isolates were recovered from thirty (30) samples of fresh vegetables collected from the retail sites at Ile-Ife.

### 3.2 Biochemical characterization and percentage occurrence of isolates

Based on characteristics specified in Bergey's Manual of Systematic Bacteriology, *Shigella spp* and *E coli* were identified as shown in Table 1. Figure 2 shows the prevalence of *Shigella spp* and *Escherichia coli* to be 23.3% and16.7% respectively.

### 3.3 Antibiotic susceptibility pattern and relative resistance of isolates to antibiotics

Table 2 and Fig. 3 represent the antibiotic susceptibility pattern of the isolates and their relative resistance in percentage (%) to the antibiotics used. None of the *Shigella* spp and *Escherichia coli* isolates showed resistance to Ofloxacin. The susceptibility pattern of *Shigella* species was as follows; 85.71% resistance to Ceftriaxone and Gentamicin, 14.29% resistance to Ciprofloxacin, and 71.43% resistance to Tetracycline.

Similarly, *Escherichia coli* demonstrated very high resistance to Augmentin, Ceftriaxone, Tetracycliine and Gentamicin. Both isolates recorded the highest percentage resistance (100%) for Amoxicillin and Nitrofurantoin. Above all, >86% of the isolates were Multi Antibiotic Resistant (MAR).

**Table 1: Biochemical characterization of isolates**

| Isolate code | GR | Cat | Oxi | Cit | Sul | Ind | Mot | MR | VP | Manit | Sugar fermentation | | Probable Organism |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | S/B | G/$H_2S$ | |
| **FS1** | -ve | + | **-** | - | - | - | **-** | + | - | + | K/A | -/- | *Shigella* |
| **FS2** | -ve | + | **-** | - | - | + | **+** | + | - | + | A/A | -/+ | *E. coli* |
| **FS3** | -ve | + | **-** | - | - | + | **+** | + | - | + | A/A | -/+ | *E. coli* |
| **FS4** | -ve | + | **-** | - | - | - | **-** | + | - | + | K/A | -/- | *Shigella* |
| **FS6** | -ve | + | **-** | - | - | + | **+** | + | - | + | A/A | -/+ | *E. coli* |
| **FS7** | -ve | + | **-** | - | - | - | **-** | + | - | + | K/A | -/- | *Shigella* |
| **FS8** | -ve | + | **-** | - | - | - | **-** | + | - | + | K/A | -/- | *Shigella* |
| **FS9** | -ve | + | **-** | - | - | - | **-** | + | - | + | K/A | -/- | *Shigella* |
| **FS10** | -ve | + | **-** | - | - | - | **-** | + | - | + | K/A | -/- | *Shigella* |
| **FS11** | -ve | + | **-** | - | - | - | **-** | + | - | + | K/A | -/- | *Shigella* |
| **FS15** | -ve | + | **-** | - | - | + | **+** | + | - | + | A/A | -/+ | *E. coli* |
| **FS16** | -ve | + | **-** | - | - | + | **+** | + | - | + | A/A | -/+ | *E. coli* |

Legend: Cat: Catalase test; Oxi: Oxidase test; Cit: Citrate test; Sul: Sulphide test ; Ind: Indole test; Mot: Motility test; MR: Methyl Red; VP: Voges-Proskauer; S: Slant; B: Butt ; $H_2S$: Hydrogen sulphide production; G: Gas production; A: Acid; GR: Gram's reaction; + / +ve: Positive - /-ve: Negative; Manit: Mannitol.

**Table 2: Antibiotic susceptibility pattern of *Shigella* spp isolated**

| Isolates | AUG | CRX | GEN | OFL | AMX | NIT | CPX | TET | MAR index |
|---|---|---|---|---|---|---|---|---|---|
| FS1 | R | R | R | I | R | R | R | R | 0.9 |
| FS2 | R | R | R | I | R | R | R | R | 0.9 |
| FS3 | R | R | R | S | R | R | S | R | 0.8 |
| FS4 | R | R | R | S | R | R | S | S | 0.6 |
| FS6 | R | R | R | S | R | R | S | R | 0.8 |
| FS7 | R | R | R | S | R | R | S | R | 0.8 |
| FS8 | R | R | R | S | R | R | S | R | 0.8 |
| FS9 | R | R | R | S | R | R | S | R | 0.8 |
| FS10 | R | R | S | S | R | R | S | R | 0.6 |
| FS11 | R | S | R | S | R | R | S | I | 0.5 |
| FS15 | I | I | I | S | R | R | S | S | 0.3 |
| FS16 | R | R | R | S | R | R | S | R | 0.8 |
| %R | 91.7 | 83.3 | 91.7 | 0 | 100 | 100 | 16.6 | 75 | -- |
| %S | 0 | 8.3 | 8.3 | 83.3 | 0 | 0 | 83.3 | 16.7 | -- |

**Legend**:

R: Resistant S: Susceptible I: Intermediate AUG: Augmentin RX: Ceftriaxone

OFL: Ofloxacin AMX: Amoxicillin NIT: Nitrofurantoin CPX: Ciprofloxacin

TET: Tetracycline GEN: Gentamicin MAR: Multi Antibiotic Resistance

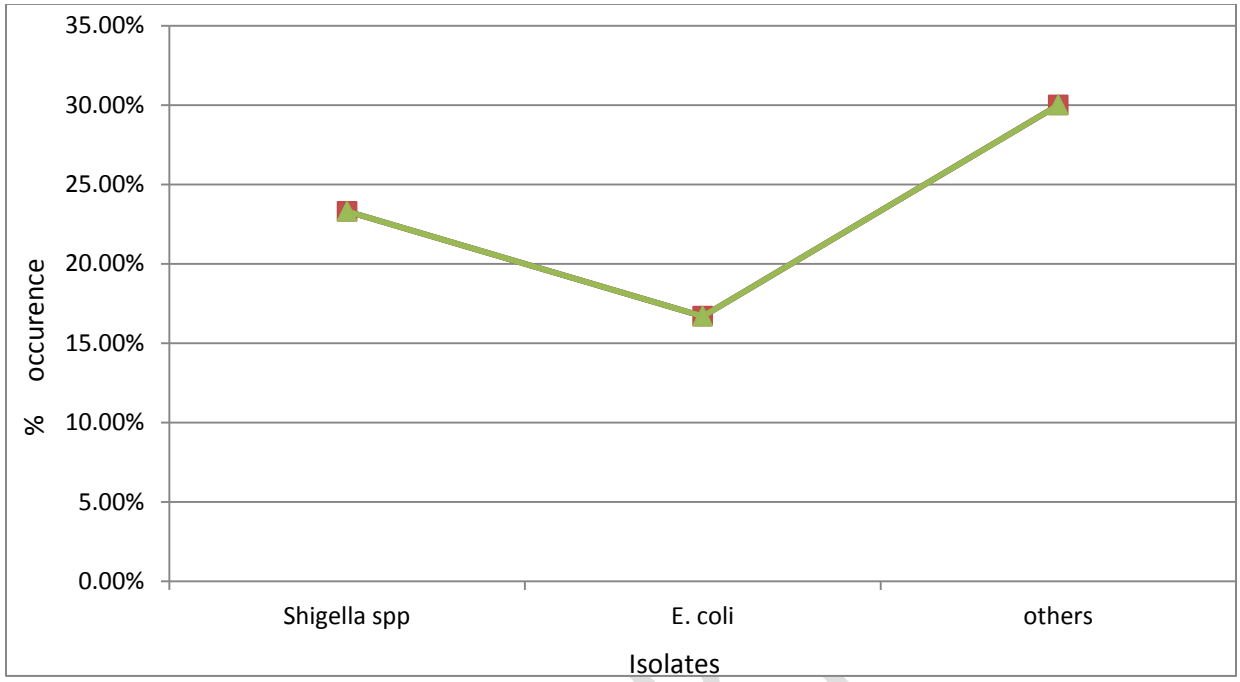


**Fig. 2: Percentage occurrence of *Shigella* spp and *Escherichia coli* isolates in the samples obtained**

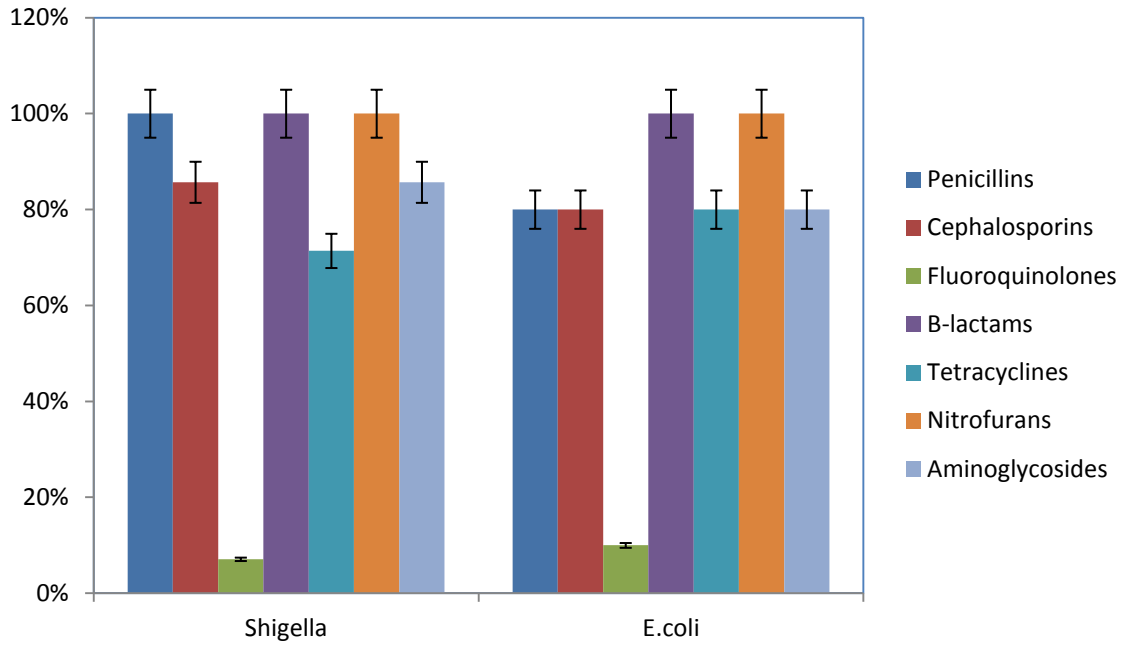


**Fig. 3: Relative resistance (%) of the isolates to classes of antibiotics.**

## 4. DISCUSSION

The isolation of pathogenic *Shigella* species and Escherichia coli from fresh and firm *Amaranthus viridis* in this study is of serious concern as these pathogens have been associated with gastroenteritis which has remained a major health care problem especially in developing and under-developed countries. [16] reported similar microbial and parasitic contamination on fresh vegetables sold in traditional markets in Hue City, Vietnam with aerobic bacteria and *Escherichia coli* (*E. coli*).

In addition, [5, 17] also reported microbial and contamination of vegetables collected from retailers in South-Western Nigeria. These pathogens in vegetables might have been a direct reflection of the sanitary quality of irrigation water for cultivation and washing/rinsing of the plant produce [18]. Although the presence of agricultural chemical residues or the presence of metals is of concern, the hazards of ready to eat vegetables reside mainly with microbial contaminants. Accounting for more than 90% of food poisoning cases each year are bacterial pathogens; *Staphylococcus aureus, Salmonella, Clostridium perfringes, Clostridium botulinum, Campylobacter, Vibrio parahaemolyticus, Bacillus cereus* and enteropathogenic *Escherichia coli* commonly found in many raw foods [5]. The presence of microorganisms in fruits and vegetables reflect the; sanitary quality of irrigation water for cultivation and washing or handlers, from the point of cultivation to the point of consumption. Therefore, vegetables might become contaminated from farms through the use of; sewage contaminated water for irrigation, organic manure as fertilizers, unclean equipment for transportation and storage, unclean cutting surfaces and equipment and unhygienic handlers [19].

The isolates were highly resistant to antibiotics in the class; penicillins, nitrofurans and β-lactams but showed little resistance to fluoroquinolones as seen in Fig. 3. This suggests the indiscriminate use of antibiotics for the prevention and control of bacterial infections and its likely disposal into nearby farmlands within the studied site. Multi Antibiotic Resistance has also been ascribed to the natural resistance of microorganisms to certain antibiotics due to the possession of drug resistant plasmids by microorganisms or acquisition of drug resistant genes via horizontal gene transfer (HGT) from other microorganisms [20].

The percentage occurrence of antibiotic resistant coliforms to commonly used antibiotics in medicine and agriculture in this study is quite worrisome as this would mean decreased therapeutic activities against bacterial infections.

## 5. CONCLUSION

This study shows that pathogenic bacteria; *Shigella* spp. and *Escherichia coli* were harbored in fresh, green vegetables (*Amaranthus viridis*) cultivated and sold in Ile-Ife, Osun State. It is important to note that despite the presence of pathogenic microorganisms in the examined vegetables, the samples did not show any visible sign of spoilage. Thus, visible appearance or organoleptic evaluation is not a good criterion for judging the microbial quality of vegetables. Inadequate cooking, improper handling and improper storage of cooked vegetables are some of the factors that could lead to presence of pathogens in cooked vegetables. Hence, application of good cooking practices and adequate food hygiene measures is essential for the prevention of food-borne pathogens in cooked vegetables. Furthermore, in cases of outbreak of enteric diseases, since the isolates were highly sensitive to ofloxacin and Ciprofloxacin belonging to the antibiotic class, fluororquinolone, fluoroquinolones should be considered as the preferred class of antibiotics in the first line of treatment.

## COMPETING INTERESTS

The authors declare no competing interests.

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