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Novel Bacteria isolated from Fresh cut Fruits and Vegetables and their response to Commercial Antibiotics

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Abstract

Vegetables and fruits are sources of nutrients with many health benefits. The crude processing and handling methods exposes the products to microbial contamination. This study investigates the indigenous bacteria associated with the contamination of vegetables and fruits sold in Imo State, Nigeria. Multidrug resistant bacterial isolates were further characterized molecularly. Analysis was carried out using standard methods and identification with reference to standard bacteriological manuals. Bacterial counts was very high on the samples and far above recommended limits. Bacterial isolates such as *Priestia flexa, Bacillus altitudinis, Klebsiella variicola, Staphylococcus aureus* and *Pantoa ananatis* are usually soil borne and predominant on vegetation. Their significant health implications on humans, animals and plant diseases had been reported. Some of the isolates, particular, *Priestia flexa*, *Bacillus altitudinis*, *Klebsiella variicola* and *Pantoa ananatis* are novel and alien to vegetables and fruits in southeast Nigeria. *Staphylococcus aureus*, *Bacillus altitudinis* and *Priestia flexa* are dominant on all the vegetables and fruits. *Utazi*, tomato and scent, carrot and pawpaw recorded high prevalence of all the isolates. The bacterial isolates were susceptible to ciprofloxacin, ampicillin and amoxicillin clavulanate. *Staphylococcus aureus* shows very high susceptibility to 60% of the antibiotics while *Klebsiella varicola* and *Priestia flexa* exhibited resistance to 70% of the antibiotics. Application of chemical fertilizer and human manure as well as poor irrigation using contaminated wastes water could be the vehicle in the transmission of these microorganisms. It is evident that contaminated fruits and vegetables have been implicated in many foodborne outbreaks throughout the world. These foodborne outbreaks are not only a burden on public health but also cause heavy economic loss to the food industry. Therefore, good manufacturing and processing practices becomes inevitable to obtain wholesome produce acceptable to ensure consumers' satisfaction.

Keywords: fruits, vegetables, bacteria, molecular studies, MDR

Introduction

Fresh-cut products are any fruits or vegetables that have been trimmed and/or peeled and/or cut and bagged or pre-packaged to offer consumers high nutrition, convenience, and flavour while still maintaining its freshness (Hurst, 2002).Fresh-cut produce on the market in some developed counties include melons, cantaloupe, watermelon, mango, jackfruit, papaya, grapefruit, pineapple, fruit mixes, shredded leafy vegetables and salad mixes, vegetables for cooking like peeled baby carrots, baby corn, broccoli and cauliflower florets, cut celery stalks, shredded cabbage, cut asparagus, cut sweet potatoes and many more (Piano and Castillo-Israel, 2019).

Microbes are found all over the globe with some few exceptions, including sterilized surfaces. They include normal flora that is nonpathogenic, which contribute to the larger percentage, and pathogenic species which are few. Thus, many pathogenic microbes have found their way into vegetables which are a great source of a healthy diet for humans. According to (Yahia *et al*., 2019), fresh vegetables and fruits play an important role in human nutrition due to their high nutrient content of vtamins, such as vitamins B, C, K, and minerals such as calcium, potassium, and magnesium, as well as dietary fibre. Fresh fruits and vegetables provide a healthy and balanced diet and can prevent chronic diseases such as heart diseases, cancer, diabetics, and obesity including several micronutrient deficiencies especially in developing countries (Septembre-Malaterre *et al*., 2018). Vegetables consumed raw are increasingly being recognized as important vehicles for the transmission of human pathogens (Berger *et al*., 2010). As fresh vegetables are eaten raw or slightly cooked to preserve the taste and their nutrient contents, this serves as a potential source of various food-borne infections and disease outbreaks (Tournas, 2005). While there is an increase in global consumption of fresh fruits and vegetables, this is greatly threatened by an upsurge of microbial contamination (Snyder and Worobo, 2018). The growing demand for vegetables has necessitated larger production. The larger production of vegetables within the shortest possible time to meet the growing demand has placed them at a higher risk of contamination with the pathogenic microbes, making the safety of consumers uncertain. Study of sources of contamination and type of pathogenic etiological agents isolated from vegetables includes *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium botulinum*, *E. coli* 0157: H7, *Listeria monocytogenes*, *Salmonella* spp., *Shigella, Staphylococcus* and *Vibrio cholerae*(Sperber and Doyle, 2009; Jay *et al*., 2005).

Vegetables are a vital component of the human diet, providing essential nutrients and contributing to overall health. However, they are also potential reservoirs of various microorganisms, including bacteria that can have both positive and negative effects on human health. While beneficial bacteria can play a role in enhancing nutrient availability and promoting gut health, pathogenic bacteria can pose risks to consumers, leading to foodborne illnesses (Jay *et al*., 2005; Snyder and Worobo, 2018).

This report investigates the indigenous multidrug resistant bacteria isolated from fresh cut vegetables and fruits sold in Owerri, Imo State, Nigeria.

Materials and Methods

Collection of Sample

Fresh vegetables and fruits were bought from four popular daily markets (Relief market, Ekeonuwa market, Cluster Market and Ihiagwa market) located in Owerri, Imo State, Nigeria. Samples were bought from different vendors in the market. Samples were fresh without any bruises or abrasion. All sample were carefully labeled and taken to the laboratory for immediate analysis. Sample size was determined according to the method of Kothari (2004).

Sample Preparation and Microbiological Analysis

Twenty grams (20 g) each of the fruits and vegetables were grind in a stomacher blender into paste in 180 ml of sterile distille water (DW). One milliliter (1 ml) of the paste was decimally transfer in bijou bottles containing sterile 9 ml physiological saline until the desired dilution is obtained. One-tenth milliliter (0.1 ml) portion of the 6^{th} dilution was inoculated into Nutrient and Eosin Methylene Blue Agar, while the same volume from the 4^{th} dilution was transferred into the remaining media. Inocula were spread evenly with a sterile glass hockey stick-like spreader and incubated at ambient temperature for 24-48 h (Ezeonu *et al*., 2013**;**Sharma, 2000; Cheesbrough, 2000; Beisher, 1987).

Enumeration, Characterization and Identification of Bacterial Isolates

Bacterial population was determined using a digital colony counter and total colonies expressed as colony forming units per milliliter/gram (Cfu/ml/g). Microbial isolates were characterized based on cultural (colonial), microscopic and biochemical methods with reference to standard manuals. The identities of the isolates were cross-matched with reference to standard manuals for the identification of bacteria (Buchanan and Gibbon, 2000; Harrigan and McCance, 2000).

Toxicity Testing of Isolates

Toxicity test of the bacterial isolates was determined by streaking 24 h old culture of the organisms on freshly prepared surface dried Blood agar and Columbia agar and incubated at 37^0C for 24-48 h. Haemolysis of red blood cells on the agar is positive for toxicity test. The degree of haemolysis determined the level of toxicity of the isolates (Pazhani *et al*., 2014).

Determine multi drug resistant (MDR) bacteria isolated from the vegetables and fruits

Twenty-four hours old pure cultures of test isolates were washed in sterile distilled water and standardized using McFarland method with cell turbidity equivalent to 1.5 x 10^8 Cfu/ml (Cheesbrough, 2000).

Standardized pure cultures of test isolates were spread evenly on a freshly prepared and surface dried Mueller Hinton Agar medium and allowed to stand for 30 mins. Five commercial antibiotics (oxoid) of known concentrations were placed at equal distances on the medium previously seeded with the test organisms. The entire set was incubated at 37^0C for 24-72 h. Zone of inhibition in millimeter (mm) was measured in triplicates and the mean recorded (Cheesbrough, 2000).

Antibiotics Susceptibility Test

Antibiotics such as tetracycline, ciprofloxacin; gentamycin; erythromycin; amoxicillin clavulanate; cephalexin; lincomycin; ofloxacin; ampicillin; chloramphenicol of known concentrations was chosen based on their narrow and broad spectrum as described by FMARDEH. (2017). Antimicrobial use and Resistance in Nigeria: Situation analysis and Recommendations.

Molecular Characterization of Isolates from Samples

Molecular characterization and extraction of DNA from the selected bacteria isolates was carried out at Niger Delta University Biotechnology Laboratory, Amasomma, Yenegoa, Bayelsa State. The extractions were done according to standard protocols with slight modifications (Kumar *et al*., 2016; Saitou and Nei, 1987; Felsenstein, 1985; Jukes and Cantor, 1969)

Results

Tables 1 and 2 shows the mean bacterial populations of vegetables and fruits respectively. Growth was luxuriant on nutrient agar followed by mannitol salt agar and least on eosin methylene blue agar.Total counts was higher on the vegetables (Table 1) than on the fruits (Table 2). Fresh leaves of *utazi* and tomato recorded the highest count on all the media. Bacterial population was highest on carrot than other fruits

on all the bacteriological media with nutrient agar ranking the medium of choice (Table 2).

The identities of the bacterial isolates are shown in Tables 3 and 4 and Fig 1.The obtained 16s rRNA sequence from the isolates produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The 16S rRNA of the isolates showed a percentage similarity to other species at 100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolates within the genera, *Priestia, Bacillus, Klebsiella, Staphylococcus* and *Pantoa* spand revealed a closely relatedness to *Priestia flexa*, *Bacillus altitudinis, Klebsiella variicola*, *Staphylococcus aureus* and *Pantoa ananatis* (Fig. 1).

Table 1 Mean Bacterial Population of Vegetables on Bacteriological Media (n=8)

Sample codes	NA	MSA	EMBA	MCA
Lettuce	3.7×10^5 –1.22×10^7	2.8×10^4 – 4.2×10^4	1.8×10^4 – 5.6×10^4	2.8×10^4– 5.1×10^4
Scent Leaf	8.1×10^5– 1.48×10^6	7.1×10^4 – 8.0×10^0	1.1×10^4– 1.9×10^4	3.8×10^4– 4.7×10^4
Cabbage	1.4×10^5– 6.2×10^6	3.0×10^4 –6.4×10^4	1.3×10^4– 5.0×10^4	2.8×10^4– 3.3×10^4
Fresh Tomato Leaf	2.2×10^5– 1.33×10^7	5.8×10^4 – 1.11×10^5	3.5×10^4– 1.16×10^5	6.8×10^4– 1.01×10^5
Utazi Leaf	5.9×10^5– 1.56×10^7	7.7×10^4 – 1.02×10^5	2.4×10^4– 2.56×10^5	4.8×10^4– 2.06×10^5

NA, nutrient agar; MSA, Mannitol Salt Agar; EMBA, Eosin Methylene Blue agar; MCA, MacConkey Agar

Table 2 Mean Bacterial Population of Fruits on Bacteriological Media (n=8)

Sample codes	NA	MSA	EMBA	MCA
Pineapple	1.7×10^5 – 2.2×10^5	2.0×10^4 – 3.1×10^4	5.1×10^4 – 1.01×10^5	1.1×10^4– 4.4×10^4
Pawpaw	1.1×10^5– 1.8×10^5	5.5×10^4 – 1.11×10^5	2.1×10^4– 1.10×10^5	20×10^4– 3.7×10^4
Apple	2.0×10^5– 3.3×10^5	1.0×10^4 –3.1×10^4	1.3×10^4– 1.00×10^5	4.0×10^4– $5.1x 10^4$
Carrot	2.2×10^5– 1.33×10^6	1.8×10^4 – 1.26×10^5	3.7×10^4– 1.10×10^5	7.1×10^4– 1.33×10^5

NA, Nutrient Agar; MSA, Mannitol Salt Agar; EMBA, Eosin Methylene Blue Agar; MCA, MacConkey agar

Table 3 Colonial and Microscopic Characteristics of Bacterial Isolates

Bacterial Isolates	Colonial Characteristics	Microscopic Characteristics	Motility	Spore Formation	Capsule Formation
Staphylococcus sp	Small circular golden yellow colonies on NA and light yellow colonies on MSA	+S	-	-	-
Klebsiella sp	Mucoid, butyrous and slimy pink colonies on EMBA and MCA	-R	-	-	+
Bacillus sp	Dull and dry serrated flat cream colonies on NA and grey colonies on EMBA	+R	+	+	-
Pantoea sp	Dull and dry serrated and wrinkled colonies and produce yellow pigment on NA	-R	+	+	-
Priestia sp	Moist and shiny slimy mucoid cream colonies	+R	+	+	-

Table 4 Biochemical and Carbohydrate Fermentation of Bacterial Isolates

Bacterial Isolates	Cat	Oxi	Coag	NO_3	Ure	In	MR	VP	Cit	G	S	L	M
Staphylococcus aureus	+	-	+	+	+	-	-	+	-	+	+	+	+
Klebsiella varicola	+	-			+	-	-	+	+	+	-	+	+
Bacillus altitudinis	+	-	-	+	+	+	-	+	+	+	-	-	-
Pantoea ananatis	+	-	-	+	-	+	+	-	+	+	+	+	-
Priestia flexa (P. megaterium)	+	+	-	-	-	-	+	-	+	+	+	+	+

Cat, catalase; Oxi, oxidase; Coag, coagulase; NO_3, nitrate reduction; Ure, urease production; IN, Indole; MR, methyl red; VP, voges proskaeur; G, glucose; S, sucrose; L, lactose; M, maltose; Cit, citrate utilization.

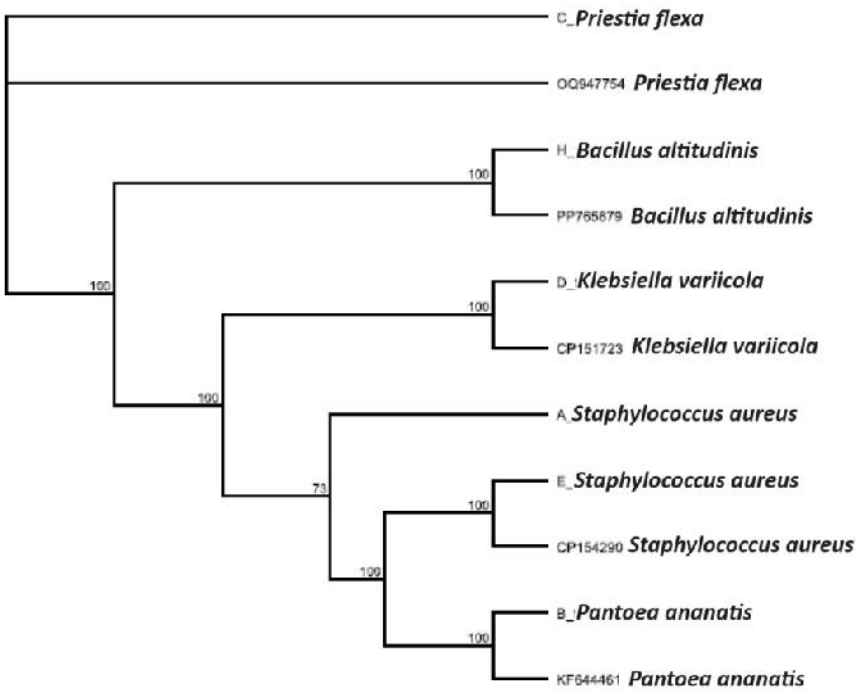

Fig 1: Phylogenetic tree showing the evolutionary relationship between the bacterial isolate

Percentage occurrence of the bacterial isolated on the vegetables and fruits is shown in Tables 5 and 6. *Staphylococcus aureus, Bacillus altitudinis* and *Priestia flexa* are dominant on all the vegetables and fruits.*Utazi*, tomato and scent, carrot and pawpaw recorded high prevalence of all the isolates.The bacterial isolates are susceptible to Ciprofloxacin, ampicillin and amoxicillin clavulanate.*Staphylococcus aureus* shows very high susceptibility to 60% of the antibiotics while *Klebsiella varicola* and *Priestia flexa* exhibited resistance to 70% of the antibiotics (Table 7).

Table 5 Percentage Occurrence of Bacterial isolates in Vegetables

Bacterial Isolates	*Utazi*	Lettuce	Cabbage	Tomato Leaf	Scent Leaf
Staphylococcus aureus	24 (21.4)	12 (13.4)	10 (13.0)	41 (28.7)	33 (20.5)
Klebsiella varicola	12 (10.7)	8 (9.0)	6 (7.8)	15 (10.5)	21 (13.0)
Bacillus altitudinis	31 (27.6)	22 (24.6)	25 32.5)	38 (26.6)	44 (27.3)
Pantoea ananatis	12 (10.7)	8 (9.0)	16 (20.8)	18 (12.6)	28 (17.4)
Priestia flexa (P. megaterium)	33 (29.4)	39 (43.7)	20 (26.0)	30 (21.0)	36 (22.3)

Number in parenthesis represent percentage (%)

Table 6 Percentage Occurrence of Bacterial isolates in Fruits

Bacterial Isolates	Carrot	Pawpaw	Apple	Pineapple
Staphylococcus aureus	33 (23.4)	21 (22.9)	8 (17.7)	41 (39.4)
Klebsiella varicola	8 (5.7)	18 (19.6)	6 (13.3)	7 (6.7)
Bacillus altitudinis	33 (23.4)	11 (12.0)	12 (26.6)	22 (21.1)
Pantoea ananatis	21 (14.9)	12 (13.1)	5 (11.1)	12 (11.5)
Priestia flexa (P. megaterium)	45 (32.0)	30 (32.7)	14 (31.1)	22 (21.1)

Number in parenthesis represent percentage (%)

Table 7 Antibiotic Sensitivity of Profile of Bacterial Isolates (n=3)

Bacterial Isolates	TE	CIP	GEN	ERY	AMC	CEP	C	LIN	OFX	AMP
Staphylococcus aureus	S	S	S	S	S	R	R	R	R	S
Klebsiella varicola	R	S	R	R	S	R	R	R	R	S
Bacillus altitudinis	R	S	R	R	S	S	S	R	R	S
Pantoea ananatis	S	S	R	R	S	R	R	R	R	S
Priestia flexa (P. megaterium)	R	S	R	R	S	R	R	R	R	S

TE, tetracycline; CIP, ciprofloxacin; GEN, gentamycin; ERY, erythromycin; AMC, amoxicillin clavulanate; CEP, cephalexin; LIN, lincomycin; OFX, ofloxacin; AMP, ampicillin; C, chloramphenicol. Results in CLSI Standard.

Discussion

Fruits and vegetables can be consumed in its raw form without undergoing processing or conversion (Saka *et al*., 2022; Garba *et al*., 2021; Balali *et al.,* 2020).Foodborne diseases caused by bacterial contamination of vegetables remain a significant public health concern worldwide. Outbreaks linked to contaminated vegetables had been documented (Yi *et al*., 2023;Saka *et al*., 2022;Garba *et al*., 2021; Balali *et al.,* 2020; Hussain and Gooneratne, 2017; Ovais *et al*., 2016; Skara and Rosnes, 2016). Characterization and identification of bacteria associated with vegetables are essential steps towards implementing food safety measures and minimizing the risk of foodborne illnesses.

The larger production of vegetables within shortest possible time to meet the growing demand has placed them at risk of contamination with pathogenic microorganisms, making the safety of consumers uncertain. The sources of contamination of vegetables and fruits are varied and include the application of organic wastes to farm as fertilizer, contamination of water used for irrigation with faecal material, direct contamination by livestock,wild animals and birds and post-harvest issues such as workers hygiene (Rajwar *et al*., 2016; Heaton and Jones, 2008). Human activities cannot be completely separated from microbes, thus many pathogenic microbes find their way into vegetables and fruits which are a great source of a healthy diet for humans (Yahia *et al*., 2019). Vegetables and fruits consumed raw are increasingly being recognized as important vehicle for the transmission of human pathogens (Snyder and Worobo, 2018). Snyder and Worobo (2018)and Yahia *et al*.(2019) had independentlyreported the presence of *Staphylococcus aureus, Shihella, Salmonella, Vibrio, Listeria monocytogenes, Campylobacter jejuni, Clostridium botulinum* and *Escherichia coli* 0157:H7 in vegetables.

Previous studies have focused on identifying bacterial species on various foodsources, including fruits and vegetables adopting culture-based methods. This study identified bacterial species using conventional and molecular techniques such as polymerase chain reaction (PCR) and DNA sequencing which have revolutionized the field of microbial ecology, enabling more accurate and comprehensive analysis of bacterial communities.*Staphylococcus aureus, Bacillus altitudinis, Klebsiella varicola, Pantoea ananatis* and *Priestia flexa* were the predominant isolates from the fruits and vegetables studied.

These microorganisms are frequently isolated from soil and plants with attendant pathogenicity inimmunocompromised individuals, though in some the disease is usually self-limiting. *B. altitudinis* is ubiquitous and frequently isolated from plant (root and leaf), rhizosphere soil, soil and animals (Wang *et al*., 2021). The toxins produced by infected tubers are potentially harmful to humans (Ellner, 2002).*P. flexa* previously belonging to *Bacillus* (*Bacillus flexa*) and sharing a close relationship evolutionarily with *B. cereus* and *B. subtilis* is prominent in the soil and inner tissues of plants of healthy cotton plants (Patel and Gupta, 2020; Gupta *et al*., 2020). Deswal *et al*. (2023) isolated a new strain, *Priestia flexa* from human faeces implicated in mucin degradation.This bacterial species may be also be isolated from faeces of poultry, but human pathogenicity has not been well described yet.
Pantoea species commonly isolated from humans is widely distributed in nature and has been isolated from numerous ecological niches, including plants, water, soil, humans and animals. *P. ananatis* is capable of infecting humans and are found in diverse ecological niches, such as bacterial community contaminating aviation fuel tanks and contribute to the growth promotion of potato and pepper (Coutinho and Venter, 2009). *P. ananatis* infects both monocotyledonous and dicotyledonous plants with diverse symptoms ranging from leaf blotches and spots; die-back; and stalk, fruits and bulb rot. It also has antibacterial and antifungal properties (Morin, 2014).*P. ananatis* has been reported to cause bloodstream infection (BSI), including neonatal

BSI related to the administration of contaminated intravenous solution or to contamination of indwelling catheters, septic shock, respiratory failure and death in contaminated parenteral fluid (Hunt and Sophonie, 2023). *Klebsiella* is a common opportunistic pathogen for humans and other animals, as well as being resident or transient flora, particularly in the gastrointestinal tract. Other habitats include sewage, drinking water, soils, surface waters, industrial effluent and vegetation (Bagley, 1985).*Klebsiella varicola* is an emerging pathogen in humans and has been described in different environment (Barrios-Camacho *et al*., 2019). The authors also reported the crossing bacteria from plants, thus, establishing this as a possible route of transmission.Cucumbers and carrot had been reported as highest contaminated leafy green vegetables by multidrug resistant *Staphylococcus aureus*(Jia *et al*., 2023).

Fresh-cut fruits and vegetables have a higher microbial risk profile than the 'whole' produce (Husein and Gooneratne, 2017). Fresh cut fruits and vegetables mostly have a higher risk of contamination during preparation, packaging and storage (Wills and Golding, 2016; Balali *et al*, 2020). Fresh-cut fruits and vegetables are more susceptible to spoilage and can facilitate rapid growth of spoilage microorganisms as well as microorganisms of public health significance (Alegbeleye *et al*., 2022; Ovais *et al.*, 2016).The destruction of surface cells during processing (such as peeling, slicing and shredding) of fresh-cut produce exposes the cytoplasm and provides microorganisms with a richer source of nutrients as compared to intact produce (Rico *et al*., 2007). Also, the high water activity and approximately neutral (vegetables) or low acidic (many fruits) tissue pH facilitate rapid microbial growth (Sperber and Doyle, 2009). These conditions provide a perfect platform for a number of important human pathogens and spoilage microorganisms to contaminate fresh-cut produce, which results in a faster deterioration of fresh-cut produce compared to whole fruit or vegetable.

Conclusion and Recommendation

Fruits and vegetables collectively referred to as fresh produce are important components of a healthy diet. Fruits and vegetables are rich sources of vitamins and minerals, dietary fibre and a host of beneficial non-nutrient substances including plant sterols, flavonoids and other antioxidants. Fruits and vegetables rich diet is well established for its e ciency to promote human health, in particular to regulate the body weight, help reduce the risk of coronary heart disease, stroke and certain types of cancer.

Biological hazards in fresh produce come from microorganisms such as bacteria, fungi (yeasts and moulds), protozoans,viruses and helminthes (worms). Biological contaminants, particularly bacteria constitute the leading cause of foodborne illnesses. The five bacteria isolated from the vegetables and fruits under investigation are potential contaminants residents in soil, water and vegetation. These sources serves as the major vehicle in the transmission of the microorganisms. It is evident that contaminated fruits and vegetables have been implicated in many foodborne outbreaks throughout the world. These foodborne outbreaks are not only a burden on public health but also cause heavy economic loss to the food industry. Therefore, good manufacturing and processing practices remains the *sine qua non* in the production of wholesome and acceptable produce to ensure consumers' satisfaction.

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