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Prevalence of Enterobacteriaceae in Fresh Vegetables Sold in Open Markets in Benin City Nigeria

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ABSTRACT: Vegetables are good sources of nutrients and can be contaminated by bacteria thereby causing spoilage and food-borne illnesses. The aim of this study was to determine the prevalence of Enterobacteriaceae in fresh vegetables sold in open markets in Benin City, Edo State. Thirty fresh vegetables samples comprising of scent, utazi, uziza, pumpkin and okazi leaves were obtained from three open markets. Isolation was carried out using pour plate technique and isolates were identified based on cultural, morphological and biochemical characteristics. The isolates were subjected to acid and bile salt tolerance test at pH 3.0 and 0.3 % bile salt concentration respectively. The Enterobacteriaceae count ranged from $0.02 \pm 0.01 \times 10^6$ cfu/g (Uselu market) to $2.01 \pm 0.04 \times 10^6$ cfu/g (New Benin market). The identified isolates were *Proteus mirabilis*, *Salmonella* spp., *Escherichia coli*, *Enterobacter aerogenes* (PUO), *Citrobacter freundi*, *Klebsiella pneumoniae* and *Enterobacter aerogenes* (UTN). The isolate *E. aerogenes* (PUO) had the highest occurrence of 22.94 % while *P. mirabilis* had the lowest occurrence of 6.42 %. Results of the acid tolerance test showed that *Enterobacter aerogenes* (UTN) had the lowest percentage viability of 81.43% while *Salmonella* spp. had the highest percentage viability of 100% after 3 h of incubation. *Salmonella* spp. had the lowest percentage viability of 87.80% while *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter aerogenes* (PUO) and *Citrobacter freundii* had the highest percentage viabilities of 100% for bile salt tolerance, after 3 h of incubation. The high bacterial counts in this study could be attributed to poor hygienic practices at the markets. Therefore, proper public education on good food handling practices in markets is advised.

Keywords: Fresh vegetables, Enterobacteriaceae, Open markets, Foodborne illness

Introduction

Vegetables are rich dietary sources of micronutrients; minerals, vitamins and most importantly, antioxidants and fiber, which are vital to human health, wellbeing and disease prevention (Eni *et al*., 2010). As providers of vitamins (A, C, B6, niacin, and thiamin), minerals (such as calcium, magnesium, iron, and phosphorus), fresh vegetables make a significant contribution to human nutrition (Lawal *et al*., 2015). Vegetables can be found as fruits, seeds, or leaves. *Vernonia amygdalina* (bitter leaf), *Talinum triangulare* (water leaf), *Telfairia occidentalis* (fluted pumpkin leaf), *Gnetum africanum* (okazi or ukazi leaf), *Ocimum gratissimum* (scent leaf), *Piper guineense* (uziza leaf), and *Gongronema latifolium* (utazi leaf) are among the leafy vegetables that are frequently consumed in Nigeria (Wilson, 2008). Vegetables are recognized as roughages that promote digestion and aid to prevent constipation; they are useful in neutralizing acid substances created in the process of digestion of meat, cheese, and other foods. It has been claimed that vegetables constitution, which includes flavonoids, carotenoids, polyphenols, and phytonutrients, lowers the risk of diseases like cancer (Tafinta *et al*., 2013).
Vegetables can act as reservoirs for pathogenic or opportunistic pathogens. There are numerous pathogenic microorganisms found in fresh vegetables, including *Salmonella* spp., *Shigella* spp., enterotoxigenic *E. coli* (ETEC), shigatoxigenic *E. coli* (STEC), *Listeria monocytogenes*, *Vibrio* spp., *Campylobacter* spp., *Bacillus cereus*, *Yersinia enterocolitica*, *Clostridium botulinum*, and some viruses and parasites (Beuchat, 2002). Some of these disease-causing microorganisms may contaminate the vegetables on the farm, through contact with

organic wastes (feces, organic manure and sewage) used as nutrients or composts to improve soil quality and untreated irrigation water. This has typically been the cause of food-borne infections (Ozlem, 2005). The aim of this study was to determine the prevalence of Enterobacteriaceae in fresh vegetables sold in open markets in Benin City.

Materials and methods

Sample collection: A total of 30 samples of fresh leafy vegetables which include; scent leaf (*Ocimum gratissimum*), fluted pumpkin leaf (*Telfairia occidentalis*), utazi leaf (*Gongronema latifolium*), uziza leaf (*Piper guineese*) and okazi leaf (*Gnetum africanum*) were obtained from three open markets, namely; New Benin, Oba and Uselu markets in Benin City, Edo State. All samples were collected in sterile polythene bags and taken immediately to the laboratory for analyses.

Isolation and identification of bacteria: Each vegetable sample was chopped into small sizes with a sterile scissors. 1.0 g of each sample was homogenized in 9.0ml of peptone water. Thereafter, the samples were serially diluted and 0.1 mL of dilution 10^{-2} was inoculated on MacConkey agar in sterile Petri dishes. The inoculated plates were incubated at 37 °C for 24 to 48 h. Colonies were counted and recorded as colony forming unit per gram (cfu/g). Pure cultures were obtained by sub-culturing on fresh agar plates. Bacterial isolates were identified by morphological and biochemical characteristics (Cheesebrough, 2006).

Acid tolerance: A 2 mL of overnight grown cultures of isolates were centrifuged at 4000 rpm for 20 min using a centrifuge and the cellular pellets were re-suspended in sterile distilled water in tubes. Thereafter, isolates were standardized using 0.5 McFarland turbidity standard to a cell density of 1.5 x 10^8 cfu/ml and inoculated in 10 mL of MacConkey broth previously adjusted with 5N HCl to pH 3. Bacterial isolates inoculated in MacConkey broth without any bile salt served as the control for the experiment. The inoculated cultures were incubated at 37 °C for a period of 1 and 3 h. 0.1 mL of each broth culture were collected and spread onto MacConkey agar plates using a glass rod, then incubated for 24 h at 37 °C (Hassanzadazar *et al*., 2012; Nawaz *et al*., 2017).

The total number of viable bacterial cells were enumerated and the percentage of growth was calculated using the equation below:

$$\text{Viability (\%)} = \frac{Nt}{No} \times 100$$

where Nt = log cfu at interval 1 and 3 h
No = log cfu of control

Bile salt tolerance: A 2 ml of overnight grown cultures of isolates were centrifuged at 4000 rpm for 20 min using a centrifuge and the cellular pellets were re-suspended in sterile distilled water in tubes. Thereafter, isolates were standardized using 0.5 McFarland turbidity standard to a cell density of 1.5 x 10^8 cfu/ml and inoculated in 10 ml of MacConkey broth previously adjusted with bile salt (cholic acid) at a concentration of 0.3%. Bacterial isolates inoculated in MacConkey broth without any bile salt served as the control for the experiment The inoculated cultures were incubated at 37 ˚C for a period of 1 and 3 h. 0.1 mL of each broth culture were collected and spread onto MacConkey agar plates using a glass rod, then incubated for 24 h at 37 ˚C (Hassanzadazar *et al*., 2012; Nawaz *et al*., 2017).

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where Nt = log cfu at interval 1 and 3 h
No = log cfu of control

Results

Table 1 shows the total Enterobacteriaceae counts (cfu/g) of fresh leafy vegetables sold in Benin City, Edo State. It was observed that scent leaf samples from Uselu market had the lowest count of $0.02 \pm 0.01 \times 10^6$ cfu/g while the pumpkin leaf samples from New Benin market had the highest count of $2.01 \pm 0.04 \times 10^6$ cfu/g.

Table 1: Total Enterobacteriaceae counts (x 10^6cfu/g) of fresh vegetables sold in open markets in Benin City

Leaf	Market		
	New Benin	**Oba**	**Uselu**
Scent	1.33 ± 0.43	0.66 ± 0.06	0.02 ± 0.01
Utazi	1.96 ± 0.04	0.62 ± 0.03	1.86 ± 1.40
Uziza	1.84 ± 0.29	1.53 ± 0.55	1.20 ± 0.25
Pumpkin	2.01 ± 0.04	0.92 ± 0.60	1.67 ± 1.15
Okazi	0.92 ± 0.80	0.05 ± 0.02	0.16 ± 0.13

Table 2 shows the identified Enterobacteriaceae isolates and percentage frequency of occurrence in the fresh vegetable. The identified isolates were *Proteus mirabilis, Salmonella* spp., *Escherichia coli*, *Enterobacter aerogenes, Citrobacter freundii* and *Klebsiella pneumoniae.* It was observed that *P. mirabilis* had the lowest frequency of 6.42 % and *E. aerogenes* (1) had the highest frequency of 22.94 %.

Table 2: Identified bacterial isolates and frequency of occurrence of in fresh vegetables

Bacteria Isolates	Percentage Occurrence (%)
Proteus mirabilis	6.42
Salmonella spp.	11.01
Escherichia coli	18.35
Enterobacter aerogenes (1)	22.94
Citrobacter freundii	11.93
Klebsiella pneumoniae	22.02
Enterobacter aerogenes (2)	7.34

Table 3 shows the acid tolerance of the Enterobacteriaceae isolates at pH 3. This ranged from 90% (*Escherichia coli*) to 100% (*Proteus mirabilis* and *Klebsiella pneumoniae*) after 1 h of incubation and 81.43% [(*Enterobacter aerogenes* (2)] to 100% (*Salmonella* spp.) after 3 h of incubation.

Table 3:% Viability of Enterobacteriaceae Isolates at pH 3

Isolate	% Viability	
	1 h	**3 h**
Proteus mirabilis	100.00	82.41
Salmonella spp.	96.90	100.00
Escherichia coli	90.00	88.63
Enterobacter aerogenes (1)	98.37	83.98
Citrobacter freundii	98.14	92.11
Klebsiella pneumoniae	100.00	98.80
Enterobacter aerogenes (2)	91.20	81.43

Table 4 shows the results of the bile salt tolerance test for each Enterobacteriaceae isolate at 0.3% bile salt concentration. This ranged from 87.96 % [*Enterobacter aerogenes* (2)] to 100% (*Proteus mirabilis*, *Escherichia coli* and *Klebsiella pneumoniae*) after 1 h of incubation and 87.80 % (*Salmonella* spp.) to 100 % (*Escherichia coli*, *Enterobacter aerogenes* (1), *Citrobacter freundii* and *Klebsiella pneumoniae*) after 3 h of incubation.

Table 4: Bile salt tolerance of Enterobacteriaceae isolates in 0.3% concentration of bile salt

Isolates	% Viability	
	1 h	**3 h**
Proteus mirabilis	100	95.60
Salmonella spp.	88.81	87.80
Escherichia coli	100	100
Enterobacter aerogenes (1)	99.53	100
Citrobacter freundii	93.74	100
Klebsiella pneumoniae	100	100
Enterobacter aerogenes (2)	87.96	89.10

Discussion

In this study, the prevalence of Enterobacteriaceae in fresh vegetables sold in open markets in Benin City, Edo state was determined. The bacterial counts reported in this study could be attributed to the unhygienic practices at the markets such as improper handling and washing of vegetables with contaminated water (Al-Holy *et al.*, 2013).

Six species of bacteria were isolated from the vegetable samples. They include *Proteus mirabilis*, *Salmonella* spp., *Escherichia coli*, *Enterobacter aerogenes*, *Citrobacter freundii* and *Klebsiella pneumoniae*. This result corresponds to the findings of Erinle and Ajayi (2022) who isolated *Proteus mirabilis* and *Salmonella* spp. from fresh leafy vegetables. Also, Al-Holy *et al*. (2013) isolated *Citrobacter freundii*, *Enterobacter aerogenes*, *Escherichia coli* and *Klebsiella pneumoniae* from fresh leafy green vegetables sold in Saudi Arabia. The fresh vegetables may have been contaminated prior to or at the point of sales in the market. The soil is one of the most important reservoirs of many microbes. It also contributes to the increased contamination of fresh vegetables and the presence of bacteria such as *Enterobacter aerogenes* which were found present in this study.

Escherichia coli are often used as an indicator for fecal contamination, since the organism mostly emanate from the intestinal tract of humans and animals. The presence of *Escherichia coli* in this study could be attributed to the problem of utilizing untreated manure as soil fertilizers in the field or due to the use of contaminated irrigation water (Aycicek *et al*., 2006). *Escherichia coli* has the ability to colonize and internalize mainly in fresh vegetables. It can be retained by several parts of the plant such as leaves even after vigorous washing or disinfection. All isolates from this study are members of the Enterobacteriaceae family. *Escherichia coli*, *Citrobacter freundii, Enterobacter aerogenes* and *Klebsiella pneumoniae* belong to the coliform group. Members of the coliform group originate from the intestine of warm-blooded animals while some others inhabit the soil, water and plant material. The presence of coliforms in food is an indication of possible fecal contamination and reflects the hygiene standard of food. Improper handling and storage can allow the level of contamination to increase. Their presence in foods also indicate that circumstances are suitable for the presence of other enteric pathogens. *Salmonella* and *E. coli* have also been implicated as foodborne pathogens resulting in symptoms such as fever, abdominal cramps, diarrhea, vomiting and headache (Bintsis, 2017).

Acid resistance is an indication of the potential of an organism to survive the gastric and duodenal juices. The increase in viability rather than decline demonstrated by *Salmonella* sp. after 1 and 3 h of incubation shows that it will take a longer exposure for an acid stressed environment to affect its growth. However, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Escherichia coli*, *Citrobacter freundii* and *Enterobacter aerogenes* experienced a decline in viability after 1 h of exposure. This implies the inability of the strains to adjust to the acid stressed environment.

Bile salt tolerance is also an important parameter to evaluate the survival of microorganisms in the small intestine. Bacterial is growth inhibited by bile which enters through the duodenal section of the small intestine. This is possible as the bacterial cell membrane is made up of lipids and fatty acids which are sensitive to bile salts. *E. coli* and *Klebsiella pneumoniae* were highly bile-tolerant, maintaining 100 % viability after 1h and 3h incubation. *Salmonella* sp., *Enterobacter aerogenes* and *Citrobacter freundii* showed increased percentage viability after incubation. However, *P. mirabilis* showed a decline in percentage viability from 100 % to 95 % after 3 h of incubation. Findings from this study reveal that these isolates have the potential to survive the acid barrier in the stomach and the bile rich environment of the small intestine of the digestive tract, consequently, leading to foodborne illnesses.

Conclusion

In this study, all fresh vegetables purchased from the three open markets, were contaminated with bacteria. Hence, the vegetables were of poor microbiological quality. The high bacterial counts and the presence of potentially pathogenic bacteria among the most common fresh vegetables sold in these markets is of great concern, as most of these vegetables are usually consumed with little or no heating. Thus, this could potentially result in food-borne illnesses in consumers if not properly washed before consumption. Therefore, there is need for a proper public education on good food handling practices (washing vegetables with salt and clean water) and strict compliance to food quality standards in all markets. Also, vegetable farmers should employ the use of pipe-borne water to irrigate their vegetables instead of water from wells and streams and vendors should observe good personal and environmental hygiene at the open markets.

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