

Molecular Identification and Prevalence of Bacterial Isolates from Vegetables Salad Purchased From Fast Food Centres in Two Local Government Areas in Port Harcourt Metropolis, Rivers State, Nigeria.

Abstract

Introduction: The importance of daily consumption of fresh fruits and vegetables in our diet cannot be over emphasised. The desire to eat a balanced diet rich in vegetables have driven some people to rely already prepared vegetables salad from fast food centres to meet the need.

Aim: The aim of this study was to identify bacteria associated with vegetables salad in Port Harcourt metropolis and determine susceptibility of isolated bacteria from food centres located in the Local Government Areas.

Study design: A total of 200 samples of already prepared vegetables salad were examined which comprises of four samples from each of five different fast food centres in each of the 10 communities in Port Harcourt metropolis.

Methodology: 10g of vegetables salad was weighed and added to 90ml of sterile distilled water and subsequent serial dilution was made by adding 1ml to 9ml of diluents up to 10^{-3} . An aliquot of 0.1ml of last dilution was plated by spread plate technique MacConkey agar, Tryptone soy agar, Salmonella Shigella agar and mannitol soy agar in duplicate. The inoculated plates were incubated at 37°C for 18-24 hrs and examined for growth. Antibigram of the isolates were carried out using Mueller Hinton agar. The isolates were subjected to molecular analysis for identification before susceptibility testing.

Results: The bacteria isolated from the vegetables salad were *Staphylococcus aureus* 114(38%), *E. coli* 93(31%), *B. cereus* 30(10%), *Pseudomonas sp.* 26(9%), *Klebsiella sp.* 12 (4%), *Alcaligenes sp.* 7(2%), *Acinetobacter* 5(2%), *Lysinibacillus* 6(2%), *Enterobacter sp.* 4(1%) and *Providencia sp.* 2(1%). The resistance genes detected in the isolates were CTX-M, SHV and VanB.

Conclusion: The microbiological quality and safety of ready to eat salad sold in Port Harcourt seems to be a challenge despite the advances in technology and awareness that has been created.

Key Words: Vegetable salad, Bacteria, Resistance genes, Port Harcourt Metropolis

Introduction

Vegetables salad is a side dish made with a combination of different raw vegetables and it is served with a dressing (1). Vegetables used for salads are fresh and require minimal processing. As a result of current studies which linked consumption of vegetables to the ability to fight certain ailments like cancer, the demand for vegetable salad has been on the increase (2). Globally, most people consume salads due to their nutritional components and the satisfaction they provide when served with other food.

Despite the nutritional values provided by fresh vegetables, they have been implicated as vehicles for transmission of food borne infections. This is mainly because they are served raw and are minimally processed (3; 4).

Reports have pointed out that pathogenic bacteria may attach to the surface of fresh vegetables and their consumption if not properly washed may cause outbreak of food borne infection (5; 6).

Some bacteria that are implicated in food borne infections by vegetables salad include *E.coli*, *Shigella sp.*, *Salmonella sp.*, *Staphylococcus aureus*, *Klebsiella*, *Pseudomonas*, *Enterobacter sp.*, and *Bacillus sp.* (6; 7; 1).

The course of treatment for food borne illness lies mainly on identification of the responsible pathogen and determining if specific therapy is available (8). Most incidents of gastroenteritis resolve on their own, requiring only fluid replacement and supportive care. Intravenous therapy may be required for more severe dehydration. Knowledge of the responsible pathogen and its antimicrobial susceptibility pattern allows the physician to initiate, change or discontinue antimicrobial therapy (8).

Antibiotics are medicines used to prevent and treat bacterial infections.

Apart from saving lives of patients, antibiotics have played an important role in major milestones in medicine and surgery (5; 9). Efficacious application of antibiotics has aided in prevention and treatment of infections that can occur in some patients who are down with some chronic diseases such as kidney infection and diabetes (5; 10).

In developing countries where sanitation is still poor, antibiotics decrease the morbidity and mortality caused by food-borne and other poverty-related infections (10).

Antibiotic resistance is the ability of bacteria to become resistant to antibiotic. Antibiotic resistance is rising to dangerously high levels in all parts of the world (10). The rate at which antibiotic resistance occur worldwide endangers the potency of antibiotics and has negatively affected medical science (10). The antibiotic resistance crisis has been attributed to the overuse and misuse of these medications, as well as a lack of new drug development by the pharmaceutical industry due to reduced economic incentives and challenging regulatory requirements (11). Worldwide, new resistance mechanisms continue to evolve thereby militating against treatment of common infectious diseases. A growing list of infections – such as pneumonia, tuberculosis, blood poisoning, gonorrhoea, and foodborne diseases – are becoming harder, and sometimes impossible, to treat as antibiotics become less effective (12). Bacteria have developed different mechanisms to render ineffective the antibiotics used against them. The genes encoding these defence mechanisms are located on the bacterial chromosome or on extrachromosomal plasmids and are transmitted to the next generation (vertical gene transfer). Genetic elements, such as plasmids, can also be exchanged among bacteria of different taxonomic affiliation (horizontal gene transfer) (13). Horizontal gene transfer by conjugation is common in nature, or in technical systems, where the density of bacteria is high and so, accordingly, is the chance of two suitable bacterial cells coming close to each other (14; 6).

Irrational use of antimicrobial growth promoters in farm animals is associated with the transmission of resistance to humans via animal products; important pathogens under consideration in this aspect are *Salmonella spp.* and *Campylobacter spp* (15).

Bacterial resistance to drugs is an increasing threat to the human community (12). In recent times the resistance of Gram negative bacteria to several classes of drug such as beta lactams, colistin, fluoroquinolones and aminoglycosides is gaining importance as it is involved in severe health complications in humans (12). In addition, many reports suggest the presence of silent antibiotic resistance genes with varying level of expression could also pose a severe threat to the public health (11).

2. MATERIALS AND METHODS

2.1 Study Area

The study was conducted in Port Harcourt, Rivers State, Nigeria from the month of July to November, 2020. Port Harcourt is the capital of Rivers State is situate on latitude 4.75°N and longitude 7° E, located in the Niger Delta Region of Nigeria. Port Harcourt is in tropical rain forest with lengthy wet season and heavy rainfall with short dry season. The temperature all through the year is relatively constant (25-28°C). The occupations of the people are fishing and farming.

2.2 Collection of Samples

Samples of ready prepared vegetables salad were purchased from twenty (20) different fast food centres located in two Local Government Areas in Port Harcourt metropolis. The choice of these local government areas are based on the fact that they harbour most fast food centres. A total of 200 samples of vegetables salad were examined, ten (10) samples each were purchased from 10 different fast food centres in each of the two Local Government Areas. The samples were transported to the laboratory in a cooler of icebags for bacteriological examinations.

2.3 Cultivation of Samples (Bacteriological Examinations)

In a sterile wide mouth glass container, 10g of purchased vegetables salad sample was added to 90 mL (neat) of already prepared sterile normal saline and dilutions made up to 10^3 by subsequently transferring 1mL of the homogenate to 9mL sterile normal saline in fresh test tube. Then 0.1 ml each of the last dilution 10^3 were plated by spread plate in duplicate with the aid of a sterile glass rod on already prepared MacConkey agar, Tryptone soya agar, Salmonella-Shigella agar. All the media were reconstituted and sterilized according to the manufacturer's instruction. The plates were incubated at 37°C for 18 – 24 hours and examined for growth.

2.4 Identification of Isolated Bacteria

Multiple tests including colonial appearance, chemical and biochemical tests were used for the preliminary identification of isolates such as: Gram's stain, motility, catalase, coagulase, indole, citrate, oxidase, methyl red, carbohydrates fermentation.

2.5 Antibacterial Sensitivity

The antimicrobial susceptibility testing of the isolates were performed using the Kirby Bauer agar disc diffusion method. In this method, a standardized quantity of each isolate matched with 0.5 McFarland solution was uniformly spread on already prepared Mueller Hinton agar

plate. The antimicrobial disks were placed on the surface of the already inoculated agar plate and incubated at 37°C for 18 to 24 hours and examined for zones inhibition. Results were interpreted as recommended by the clinical laboratory standard institute (CLSI., 2015).

The antibiotics used for susceptibility testings includes, Tarivid 10 µg/disk, Reflacine 10 µg/disk, Ampicillin 30 µg/disk, gentamicin 10 µg/disk, streptomycin 30 µg/disk, erythromycin 30 µg/disk, ciprofloxacin 10 µg/disk, levofloxacin 20 µg/disk, Ceporex 10 µg/disk, Nalidixic acid 10 µg/disk, Augmentin 30 µg/disk, Septrin 30 µg/disk, Rifampicin 20 µg/disk, Ampiclox 20 µg/disk, Chloramphenicol 30 µg/disk, Norfloxacin 10 µg/disk, Amoxil 20 µg/disk. The gram positive bacteria were tested with gram positive disc while the gram negative bacteria were tested with gram negative disc. Zones of inhibition were measured in millimetres and sensitivity was recorded based on CLSI standard (CLSI., 2015).

2.5 Molecular Identification

The test organisms were sub cultured in Luria broth and incubated at 37^{0c} for about 10hrs.

1. DNA EXTRACTION

5ml of the overnight broth culture of the bacterial isolate was put in a vortex tube then centrifuged at 14,000rpm for 3 minutes. The supernatant was decanted and the cells were re suspended in 500ul of normal saline and heated at 95^{0c} for 20 minutes in a water bath. The heated bacterial suspension was cooled on an ice bath then centrifuged for 3 minutes at 14,000rpm. 1ml of normal saline was added again, this was mixed thoroughly using a vortex machine then centrifuged. The procedure was carried out 3 times so as to obtain a pure DNA. The supernatant containing the pure DNA was transferred to a 1.5ml micro centrifuge tube and stored at 20^{0c}.

2. DNA QUANTIFICATION

The extracted DNA was quantified using the Nano drop 1000 spectrophotometer. The software of the equipment was launched by double clicking on the Nano drop icon. The equipment was initialized with 2ul of distilled water and blanked using normal saline. 2ul of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the 'measure' button.

AMPLIFICATION OF THE 16S rRNA GENES

The 16S rRNA gene of the isolates was amplified by Polymerase Chain Reaction (PCR) using bacteria universal primers 27 F: 5' - AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5' - GGTTACCTTGTTACGACTT-3'. The PCR amplification was carried out in an ABI 9700 Applied Biosystems thermal cycler at a final volume of 40ul for 35 cycles. The PCR mix included the X2 Dream taq Master mix supplied by inqaba South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.5uM and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95⁰C for 30 seconds; annealing, 52⁰C for 30 seconds; extension, 72⁰C for 30seconds for 35 cycles and final extension, 72⁰C for 5 minutes. The product was resolved on a 1% agarose gel containing ethidium Bromide at 130V for 30 minutes and visualised on a blue light illuminator.

2.6 Polymerase Chain Reaction

Detection of Resistant genes

2.6.1 Amplification of CTX-M Genes

CTX-M genes from the isolates were amplified using the CTX-MF: 5'-CGCTTTGCGATGTGCAG-3' and CTX-MR:5'-ACCGCGATATCGTTGGT-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95⁰C for 5 minutes; denaturation, 95⁰C for 30 seconds; annealing, 52⁰C for 30 seconds; extension, 72⁰C for 30 seconds for 35 cycles and final extension, 72⁰C for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 25 minutes and visualized on a UV transilluminator.

2.6.2 Amplification of SHV Genes

SHV genes from the isolates were amplified using SHV F: 5' CGCCTGTGTATTATCTCCCT-3' SHV R: 5'-CGAGTAGTCCACCAGATCCT-3'primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 30microlitres for 35

cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95°C for 5 minutes; denaturation, 95°C for 30 seconds; annealing, 56°C for 40 seconds; extension, 72°C for 40 seconds for 35 cycles and final extension, 72°C for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 25 minutes and visualized on a UV trans illuminator.

2.6.3 Van B Amplification

The Vancomycin genes (Van B) amplifications were carried out on a 9700 ABI thermal cycler at a final volume of 30ul. The PCR components included 2X master mix, the primers used were vanBF: 5'-CCCGAATTTCAAATGATTGAAAA-3' and vanBR: 5'-CGCCATCCTCCTGCAAAA-3' respectively, 2 ul of the DNA template and RNase free water. The PCR conditions were as follows, initial denaturation 95⁰C for 5min, 35cycles of 95⁰C for 30seconds, annealing 55⁰C for all the genes, extension 72⁰C for 45 seconds, final extension 72⁰C for 3 minutes.

2.7 Evolutionary Relationships of Bacteria Isolated from Vegetable Salad in Some Restaurants in Port Harcourt and Obio/Akpor

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 as shown in plate 1. 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 isolate CR1a within the *Acinetobacter* sp and revealed a closely relatedness to *Acinetobacter* sp strain Cr1, MS2, CR1, MS3 and UST2 were closely related to *Pseudomonas aeruginosa*, *Enterobacter asburiae*, *Alcaligenes faecalis* and *Staphylococcus aureus* respectively. Gen2, CR3, TP2, MS4, MS1 and TP1 were closely related to *Lysinibacillus fusiformis*, *Bacillus flexus*, *Providencia stuartii*, *E. coli*, *Pseudomonas* sp strain OBEG and *Klebsiella variicola* as shown in fig. 2.

2.8 Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using MAFFT. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analysed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969).

3.0 Results

3.1 Prevalence of Bacteria Isolated from Vegetable Salads in Some Restaurants in Port Harcourt and Obio/Akpor

Figure 1 shows the overall prevalence of the bacteria isolated from the vegetables salad. The prevalence of the isolates were as follows: *S. aureus* sp. 114 (38%), *E. coli* 93 (31%), *B. cereus* 30 (10%), *Pseudomonas* sp. 26 (9%), *Klebsiella* sp. 12 (4%), *Alcaligenes* sp. 7 (2%), *Acinetobacter* 5 (2%), *Lysinibacillus* 6 (2%), *Enterobacter* sp. 4 (1), and *Providentia* 2 (1%).

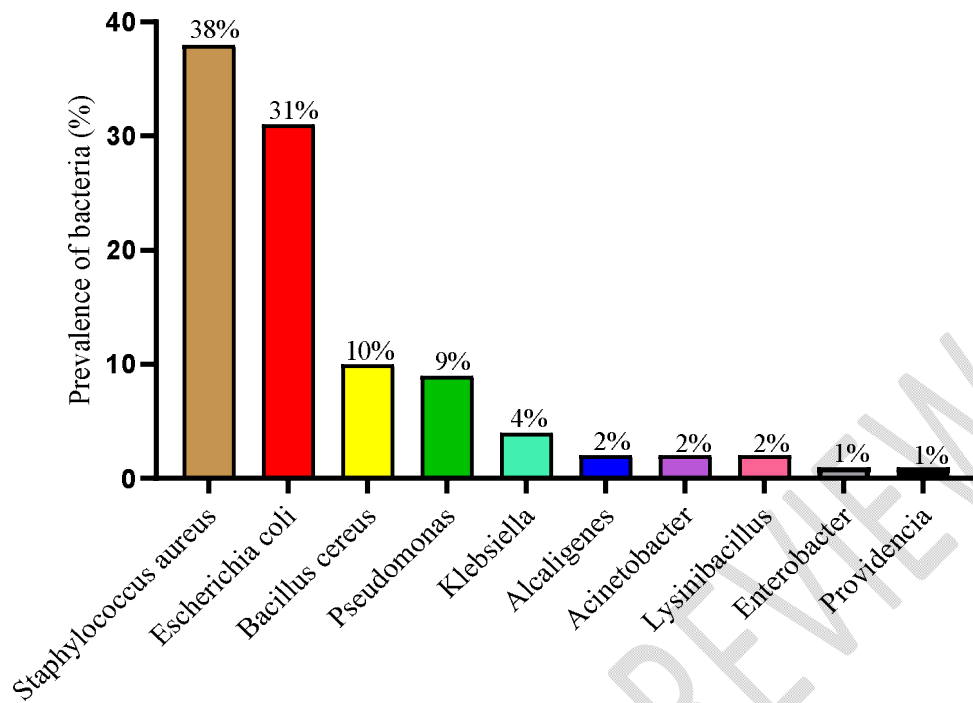


Figure 1: Prevalence of Bacteria Isolated from Vegetables Salad in Port Harcourt and Obio/Akpor

Table 3.1 shows the prevalence of bacteria detected in vegetables salad in different restaurants in Port Harcourt City local government area. The results obtained across the different communities are as follows: *Staphylococcus aureus* has a prevalence of Diobu (30%), D-Line and Elekahia (20%), Abuloma (16%), Trans-Amadi (14%), *Bacillus cereus*; Diobu (44%), D-Line (25%), Abuloma (19%), Elekahia (12%), Trans-Amadi (0%), *E. coli*; Diobu (30%), D-Line (23%), Trans-Amadi (20%), Elekahia (15%), Abuloma (13%), *Enterobacter* sp. and *Lysinibacillus* were only present in D-Line with 100% prevalence. *Klebsiella* sp. and *Acinetobacter* sp. were present in the same three location as: Diobu (50%), Abuloma (25%), D-line (25%), and Diobu (33%), Abuloma (33%), D-line (33%) respectively. *Pseudomonas* sp. was isolated in Diobu (62%), D-line (13%), Trans-Amadi (13%), Abuloma (6%), Elekahia (6%). *Providencia* sp. was found only in D-Line (50%) and Elekahia (50%).

Table 3.1: Bacteria Isolated from Vegetables Salad in PH LGA

Bacteria (n)	Abuloma	D - Line	Diobu	Trans-Amadi	Elekahia
<i>S. aureus</i> spp (50)	8 (16)	10 (20)	15 (30)	7 (14)	10 (20)
<i>Bacillus cereus</i> (16)	3 (19)	4 (25)	7 (44)	0 (0)	2 (12)
<i>E. coli</i> (40)	5 (13)	9 (23)	12 (30)	8 (20)	6 (15)
<i>Enterobacter</i> spp (1)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
<i>Klebsiella</i> spp (4)	1 (25)	1 (25)	2 (50)	0 (0)	0 (0)
<i>Pseudomonas</i> spp (16)	1 (6)	2 (13)	10 (62)	2 (13)	1 (6)
<i>Alcaligenes</i> (5)	2 (40)	3 (60)	0 (0)	0 (0)	0 (0)
<i>Lysinibacillus</i> (5)	0 (0)	5 (100)	0 (0)	0 (0)	0 (0)
<i>Acinetobacter</i> (3)	1 (33)	1 (33)	1 (33)	0 (0)	0 (0)
<i>Providentia</i> (2)	0 (0)	1 (50)	0 (0)	0 (0)	1 (50)

Key: n represent the number of the isolates

Numbers in parenthesis = percentages

Table 3.2 showed the prevalence of bacteria detected in vegetables salad in different restaurants within Obio/Akpor Local government Area. The prevalence of the bacteria isolated across the different communities are as follows: *Staphylococcus aureus* has a prevalence of Choba (28%), Elioizu (23%), Rumuodara (22%), Rumuokoro (16%), Rumuomasi (11%). *Bacillus cereus* has a prevalence of: Rumuodara (36%), Choba and Rumuokoro (29%), Elioizu (7%), Rumuomasi (0%). *Escherichia coli* had a prevalence of Rumuodara (28%), Rumuokoro (25%), Choba (21%), Rumuomasi (17%), Elioizu (9%). *Enterobacter* sp. and *Lysinibacillus* sp. were only isolated in Choba while *Klebsiella* sp. and *Providentia* sp. were not isolated from Obio/Akpor LGA. *Pseudomonas* sp has a prevalence of: Choba (50%), Elioizu (20%), Rumuodara, Rumuokoro and Rumuomasi (10%). *Alcaligenes* sp. was found only in Choba (50%) and Rumuodara (50%). *Acinetobacter* sp. was present in Elioizu (40%), Choba (40%), Rumuomasi (20%).

Table 3.2: Bacteria Isolated from Vegetables Salad Obio/Akpor LGA

Bacteria (n)	Eliozu	Choba	Rumuodara	Rumuokoro	Rumuomasi
<i>S. aureus</i> (64)	15 (23)	18 (28)	14 (22)	10 (16)	7 (11)
<i>Bacillus cereus</i> (14)	1 (7)	4 (29)	5 (36)	4 (29)	0 (0)
<i>E. coli</i> (53)	5 (9)	11 (21)	15 (28)	13 (25)	9 (17)
<i>Enterobacter</i> spp (1)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
<i>Klebsiella</i> spp (-)	-	-	-	-	-
<i>Pseudomonas</i> spp (10)	2 (20)	5 (50)	1 (10)	1 (10)	1 (10)
<i>Alcaligenes</i> (2)	0 (0)	1 (50)	1 (50)	0 (0)	0 (0)
<i>Lysinibacillus</i> (1)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
<i>Acinetobacter</i> (5)	2 (40)	2 (40)	0 (0)	0 (0)	1 (20)
<i>Providentia</i> (-)	-	-	-	-	-

Figures in bracket = percentage

Table 3.3 represents the comparison of the prevalence of bacteria isolated from vegetables salad in the two LGAs. The different of the prevalence of the bacterial isolates in Obio/Akpor and Port Harcourt LGA's are: *S aureus* sp. (56%, 44%, $p=0.0637$), *B. cereus* (46%, 54%, $p=0.6056$), *E. coli* (57%, 43%, $p=0.0500$), *Enterobacter* sp. (25%, 75%, $p=0.1573$), *Klebsiella* sp. (0%, 100%, $p=<0.0001$), *Pseudomonas* sp. (38%, 62%, $p=0.0961$), *Alcaligenes* (29%, 71%, $p=0.1088$), *Lysinibacillus* (17%, 83%, $p=0.0209$), *Acinetobacter* (40%, 60%, $p=0.5271$), and *Providentia* (0%, 100%, $p=0.0455$). There was significant difference in the percentage occurrence of some bbactreia.

Table 3.3: Comparison of the number of cases of Antibiotic Resistance in Food Poisoning Bacteria across Different antibiotics for both LGAs

Bacteria (n)	Obio/Akpor LGA	PH LGA	<i>p</i> -value
<i>Staphylococcus aureus</i> spp (114)	22.0±17.6	17.9±14.3	0.5753
<i>E. coli</i> (93)	18.3±14.1	16.2±13.5	0.7375
<i>Bacillus cereus</i> (30)	3.6±3.1	5.6±2.9	0.1567
<i>Klebsiella</i> spp (12)	0.0±0.0	4.7±3.0	0.0241
<i>Enterobacter</i> spp (4)	1.1±0.9	0.7±0.8	0.3065

Key: n represent the number of the isolates. A Chi-square analyses was performed on the data and *p*-value was considered significant at 95% confidence interval.

3.2 Antibiograms of Bacteria Isolated from Vegetables Salad in Port Harcourt

Table 3.4 shows the prevalence of the resistant antibiogram of gram-negative food-poisoning bacteria isolated from vegetable salad in Obio/Akpor LGA. Only two gram-negative foodborne bacteria were isolated from Obio/Akpor LGA, *E. coli* and *Enterobacter* spp. The prevalence of the resistant phenotype from the highest to the lowest were: *E. coli*; NA (46%) , SXT (38%), CEP (32%), PEF (26%), PN (16%), CPX (15%), CN (11%), AU (6%), S (4%) and OFX (2%); *Enterobacter* spp; SXT (75%), S (50%), PEF, PN, NA, CPX, CEP, AU (25%) and OFX and CN (0%).

Table 3.4: Resistant Pattern of Gram-Negative Bacteria in Obio/Akpor LGA

Bacteria (n)	OFX (%)	PEF (%)	PN (%)	CN (%)	S (%)	NA (%)	CPX (%)	SXT (%)	CEP (%)	AU (%)
<i>E. coli</i> (93)	2 (2)	24 (26)	15 (16)	10 (11)	4 (4)	43 (46)	14 (15)	35 (38)	30 (32)	6 (6)
<i>Klebsiella</i> spp (12)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
<i>Enterobacter</i> spp (4)	0 (0)	1 (25)	1 (25)	0 (0)	2 (50)	1 (25)	1 (25)	3 (75)	1 (25)	1 (25)

Keys: n represent the number of the isolates. OFX-Tarivid, PEF-Reflacine, PN-Ampicillin, CN-Gentamycin, S-Streptomycin, NA- Nalidixic acid, CPX-Ciprofloxacin, SXT-Septrin, CEP-Ceporex, AU-Augmentin

Table 3.5 displays the prevalence of the resistant antibiogram of gram-negative food-poisoning bacteria isolates from Port Harcourt City LGA. The antibiotics from the highest to the lowest resistance are: *E. coli* - CEP (46%), NA (33%), SXT (30) PEF (18%), PN (12%), CN (10%), AU (5%), OFX, S (3%); *Klebsiella* spp – PEF, NA (75%), OFX (67%), CPX (42%), CN, S (33%), PN, SXT (25%), CEP and AU (8%); and *Enterobacter* spp – NA, CEP (50%), OFX, CN, AU (25%), PEF, PN, S, CPX, SXT (0%).

Table 3.5: Resistant Pattern of Gram-Negative Bacteria in PH LGA

Bacteria (n)	OFX (%)	PEF (%)	PN (%)	CN (%)	S (%)	NA (%)	CPX (%)	SXT (%)	CEP (%)	AU (%)
<i>E. coli</i> (93)	3 (3)	17 (18)	11 (12)	9 (10)	3 (3)	31 (33)	12 (13)	28 (30)	43 (46)	5 (5)
<i>Klebsiella</i> spp (12)	8 (67)	9 (75)	3 (25)	4 (33)	4 (33)	9 (75)	5 (42)	3 (25)	1 (8)	1 (8)
<i>Enterobacter</i> spp (4)	1 (25)	0 (0)	0 (0)	1 (25)	0 (0)	2 (50)	0 (0)	0 (0)	2 (50)	1 (25)

Key: n represent the number of the isolates. OFX-Tarivid, PEF-Reflacine, PN-Ampicillin, CN-Gentamycin, S-Streptomycin, NA-Nalidixic acid, CPX-Ciprofloxacin, SXT-Septin, CEP-Ceporex, AU-Augmentin

Table 3.6 depicts the antibiogram of food poisoning gram-positive bacteria isolated from vegetable salad in Obio/Akpor LGA. From the two foodborne bacteria isolated, the antibiotics that showed the highest prevalence of resistance from the highest to lowest are: *S. aureus* E (48%), AML (46%), APX (21%), CPX, NB (16%), LEV (12%), RD (11%), CH (9%), CN (7%), S (0%) and *Bacillus* sp. AML (37%), RD, CH (17%), NB, E (13%), CPX, CN, S (7%), APX (3%). *Lysinibacillus* was highly resistant to Rifampicin (60%).

Table 3.6: Resistance Pattern of Gram-Positive Bacteria Isolates in Obio/Akpor LGA

Bacteria	CPX (%)	NB (%)	CN (%)	AML (%)	S (%)	RD (%)	E (%)	CH (%)	APX (%)	LEV (%)
<i>S. aureus</i> (114)	18 (16)	18 (16)	8 (7)	53 (46)	7 (6)	13 (11)	55 (48)	10 (9)	24 (21)	14 (12)
<i>Bacillus</i> spp (30)	2 (7)	4 (13)	2 (7)	11 (37)	2 (7)	5 (17)	4 (13)	5 (17)	1 (3)	0 (0)
<i>Lysinibacillus</i> (5)	0 (0)	1 (20)	0 (0)	0 (0)	2 (40)	3 (60)	0 (0)	2 (40)	1 (20)	0 (0)

Key: CPX-Ciprofloxacin, NB-Norfloxacin, CN-Gentamycin, AML-Amoxyl, S-Streptomycin, RD-Rifampicin, E-Erythromycin, CH-Chloramphenicol, APX-Ampiclox, LEV-Levofloxacin

Table 3.7 describes the antibiogram of food poisoning gram-positive bacteria isolated from vegetables salad in Port Harcourt LGA. From the two foodborne bacteria isolated, the antibiotics that showed the highest prevalence of resistance from the highest to lowest are: *S. aureus* E (39%), AML (38%), APX (17%), CPX, NB (13%), LEV (11%), RD (9%), CH (7%), CN (6%), S (5%) and *Bacillus* sp. AML (43%), E, CH (23%) NB, RD, APX (17%), CN, S (13), CPX, and LEV (10%).

Table 3.7: Resistant Pattern of Gram-Positive Bacteria in PH LGA

Bacteria	CPX	NB	CN	AML	S	RD	E	CH	APX	LEV
<i>S. aureus</i> (114)	15 (13)	15 (13)	7 (6)	43 (38)	6 (5)	10 (9)	45 (39)	8 (7)	19 (17)	11 (10)
<i>Bacillus</i> spp (30)	3 (10)	5 (17)	4 (13)	13 (43)	4 (13)	5 (17)	7 (23)	7 (23)	5 (17)	3 (10)
<i>Lysinibacillus</i> (0)	-	-	-	-	-	-	-	-	-	-

Key: CPX-Ciprofloxacin, NB-Norfloxacin, CN-Gentamycin, AML-Amoxyl, S-Streptomycin, RD-Rifampicin, E-Erythromycin, CH-Chloramphenicol, APX-Ampiclox, LEV-Levofloxacin.

Table 3.8 shows the average number of cases of antibiotic resistance of foodborne bacteria across different antibiotics for both LGAs. Obio/Akpor show the highest average number of cases of resistance to the different antibiotics for *S. aureus* (Obio/Akpor; 22.0 ± 17.6 , PH; 17.9 ± 14.3 , $p=0.5753$), *E. coli* (Obio/Akpor; 18.3 ± 14.1 , PH; 16.2 ± 13.5 , $p=0.7375$) and *Enterobacter* spp (Obio/Akpor; 1.1 ± 0.9 , PH; 0.7 ± 0.8 , $p=0.3065$) while Port Harcourt LGA has the highest number of resistance to different antibiotics in two bacteria: *E. coli* (Obio/Akpor; 3.6 ± 3.1 , PHC; 5.6 ± 2.9 , $p=0.1567$) and *Klebsiella* spp (Obio/Akpor; 0.0 ± 0.0 , PHC; 4.7 ± 3.0 , $p=0.0241$).

Table 3.8: Comparison of the number of cases of Antibiotic Resistance in Food Poisoning Bacteria across Different antibiotics for both LGAs

Bacteria(n)	Obio/Akpor LGA	PHLGA	<i>p</i> -value
<i>Staphylococcus aureus</i> (114)	22.0±17.6	17.9±14.3	0.5753
<i>E.coli</i> (93)	18.3±14.1	16.2±13.5	0.7375
<i>Bacillus cereus</i> (30)	3.6±3.1	5.6±2.9	0.1567
<i>Klebsiella sp</i> (12)	0.0±0.0	4.7±3.0	0.0241
<i>Enterobacter sp</i> (4)	1.1±0.9	0.7±0.8	0.3065

Key: n represent the number of the isolates. A Chi-square analyses was performed on the data and *p*-value was considered significant at 95% confidence interval.

Evolutionary Relationships of Bacteria Isolated from Vegetable Salad in Some Restaurants in Port Harcourt and Obio/Akpor

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 isolate CR1a within the *Acinetobacter* sp and revealed a closely relatedness to *Acinetobacter* sp strain Cr1, MS2, CR1, MS3 and UST2 were closely related to *Pseudomonas aeruginosa*, *Enterobacter asburiae*, *Alcaligenes faecalis* and *Staphylococcus aureus* respectively. Gen2, CR3, TP2, MS4, MS1 and TP1 were closely related to *Lysinibacillus fusiformis*, *Bacillus flexus*, *Providencia stuartii*, *E. coli*, *Pseudomonas sp strain OBEG* and *Klebsiella variicola* as seen in figure 4.2.

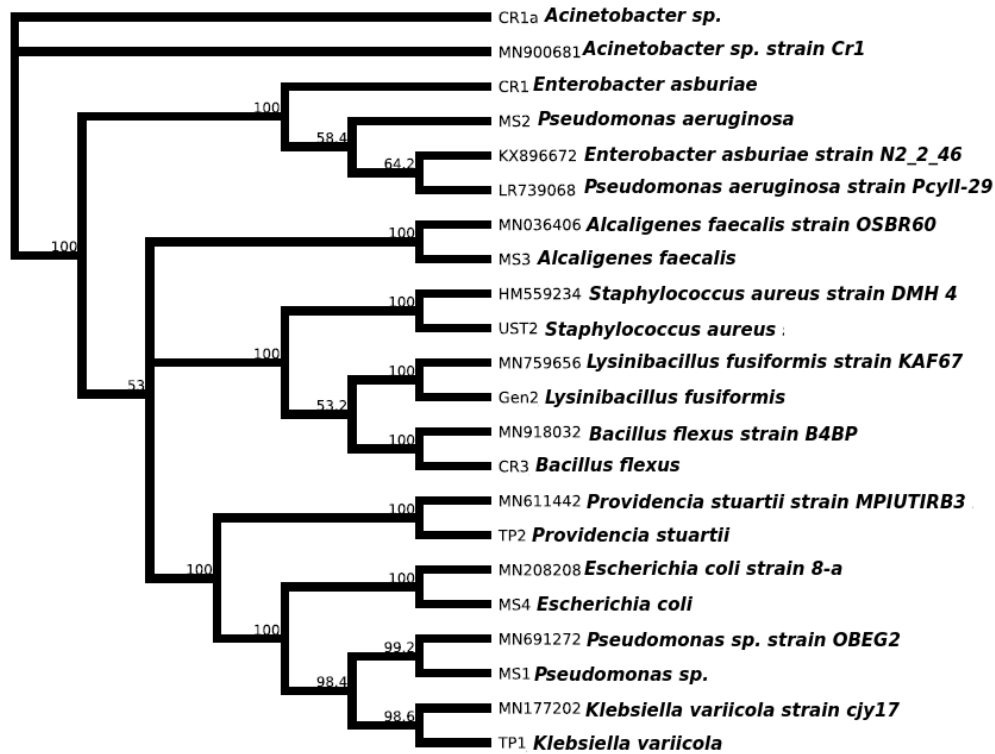


Figure 2: Phylogenetic Tree Showing the Evolutionary Distance between the Bacterial isolates. The phylogenetic tree was constructed by the Neighbour-Joining method program in Geneious software (Version 9.0.5).

Antibiotic Resistance genes

Plate 2 to Plate 4 shows the PCR amplification of the genes CTX-M, SHV and VanB genes respectively in the 12 isolates. For all the Plates, lanes 1 to 12 represent the amplification products of the different isolates. In plate 2, isolates in lanes 1, 3 and 11 were positive for the CTX-M gene. In plate 3, isolates in lanes 4, 5, 6 and 9 were positive for the SHV gene. In plate 4, isolate in lane 5 was positive for the VanB gene.

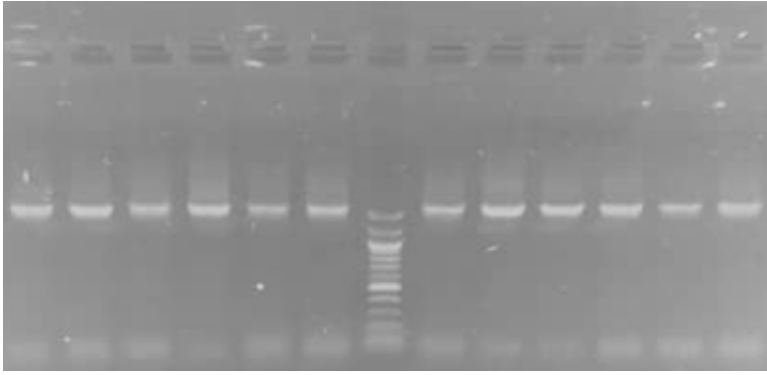


Plate 1: Agarose gel electrophoresis of the 16srRNA. Lanes 1-12 represent the 16SrRNA gene bands at 500bp while lane L represents the 100bp molecular ladder.

1 L 2 3 4 5 6 7 8 9 10 11

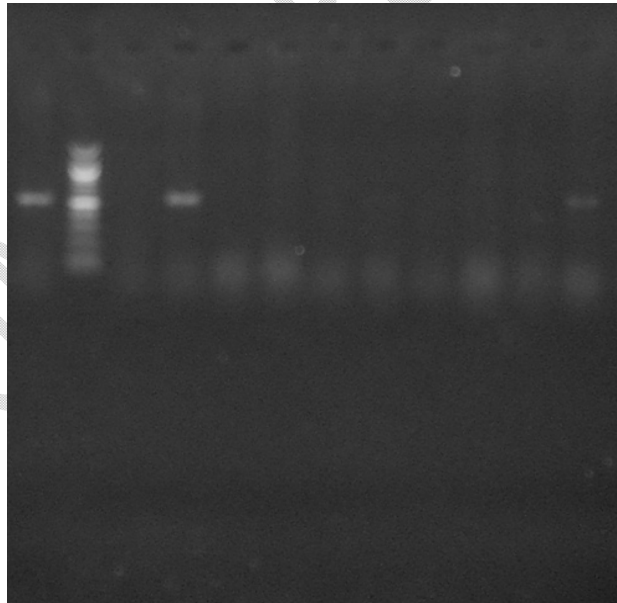


Plate 2: Agarose gel electrophoresis of the CTX-M gene. Lanes 1, 3 and 11 represent the CTX-M gene in the organisms *Providencia*, *E.coli* and *Klebsiella* at 500bp while lane L represents the 100bp molecular ladder

1 2 3 4 5 6 L 7 8 9 10 11

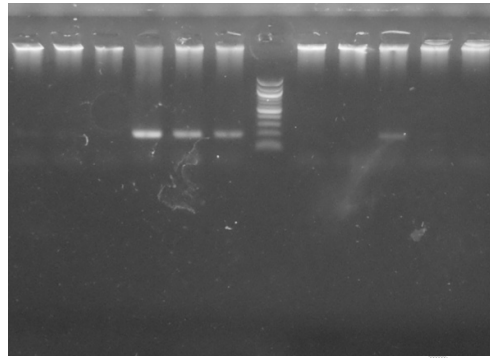


Plate 3: Agarose gel electrophoresis of the SHV gene. Lanes 4, 5, 6 and 9 represent the SHV gene in the organisms *Providencia*, *Pseudomonas*, *E.coli* and *Klebsiella* at 200bp while lane L represents the 100bp molecular ladder

1 2 3 4 5 6 7 8 9 L 10 11

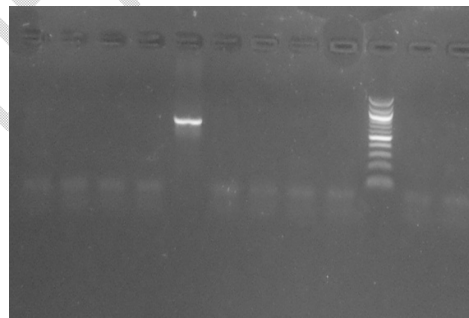


Plate 4: Agarose gel electrophoresis of the VanB gene. Lane 5 represents the VanB gene in *Staphylococcus aureus* 700bp while lane L represents the 100bp molecular ladder

DISCUSSION

The most prevalent bacteria from this research was *S. aureus* with a prevalence of 38%. This result is in line with what was obtained by Anaukwu *et al* in Awka, Anambra state in which the most prevailing bacterium from ready vegetables salad was *S. aureus* with a prevalence of 41.2% (18). However, the study conducted by Harada *et al.* (2018) in Japan, there was lower prevalence of *S. aureus* 17%. Nipa *et al* (2011) conducted a study on raw salad vegetables in Bangladesh with *S. aureus* 10.15%. The high prevalence of *S. aureus* obtained from this research calls for public health concern as *S. aureus* had been reported as the most common aetiologic agent of pyogenic infections and that staphylococcal infections may worsen some existing infections (21). In another study carried by Gbojonbola *et al* in 2012, they had a contrary report, *E. coli* was the organism most frequently recovered in vegetables salad marketed in Zaria, Nigeria (22).

In another research by Oladoyinbo *et al*, 2015 in Ogun state, it was noted that most food vendors who sell both raw and cooked food items operate recklessly without ensuring food safety precautions (23). Going by this finding, the high prevalence of *S. aureus* obtained in this study might be as a result of cross contamination by food handlers.

Escherichia coli is a normal flora of the human gastro Intestinal tract. In previous studies carried out by Wogu *et al*, 2013 in Benin City Nigeria, this bacterium was isolated from vegetable salad (35.3%) (24). Bakobie *et al*, 2017 demonstrated that the presence of *E. coli* in food samples was an indication of faecal contamination and unhealthy hygienic practices by food handlers (25). The isolation of *E. coli* in this study could be linked to unhygienic practices by the food handlers and possibly the use of unsafe water for irrigation and for processing of vegetables.

Bacillus species accounts for 10% of the isolated bacteria. This result is lower than what was recorded by Amala and Agha (2018), their result showed 18% prevalence of *B. cereus* from ready vegetables salad (3). *B. cereus* has been implicated in food poisoning and food infections (26). This could be as a result of its toxin producing ability as well as its ability to form resistant spores. The ability to form spores enables the bacteria to withstand unfavourable conditions such as low temperature obtained in refrigerators for foods preservations, especially vegetables salad. This result is contrary to the findings of Nyenje *et al.* (2012) in which *B. cereus* and *E. coli* were not isolated from vegetables samples in Alice, South Africa (27). Other organisms such as *Enterobacter sp.* (18%), *Klebsiella oxytoca* (8%), *S. aureus* (3.2%) and *Pseudomonas luteola* (2.4%) were isolated. The difference in the results obtained from these studies could be attributed to the level of public health related foodborne diseases that could be found in these different locations as well as process of preparation and personal hygiene of the vendors. Carr (2017) reported that some strains of *Pseudomonas*, *Alcaligenes*, and *Acinetobacter* could cause food spoilage and food related infections (28). The result in this study was in accordance with Abakari *et al.* (2018) and Nyenye *et al.* (2012) which demonstrated high prevalence of *Enterobacteriaceae* in vegetables and raw food (29, 27). Vegetables can be contaminated with pathogenic microorganisms during harvesting with faecal material, human handling, harvesting equipment, transport containers and transport vehicles (30).

The study considered the community distribution pattern of the bacteria isolated from the vegetables salad. This was important due to the difference in the population densities and social activities of the two LGAs. Besides *S. aureus* and *E. coli*, there were high prevalence of all the isolates in PHLGA which could be associated with the high population density and more demands for vegetables salad. The urgency to meet up demand may reduce the effectiveness of critical control points as compared to Obio/Akpor LGA.

In Obio/Akpor LGA, there was high prevalence of *S. aureus* followed by *E.coli*, while low prevalence of *Lysinibacillus* and *Enterobacter* sp. are recorded.

Comparism of bacterial isolates from the two LGAs were carried out and it showed that there was significant difference recorded for *E. coli* ($p = 0.05$), *Klebsiella* sp. ($p < 0.0001$), *Lysinibacillus* ($p = 0.0209$) and *Providencia* ($p = 0.0455$).

From the results of the antibiotic susceptibility test, the gram-negative bacteria isolated from the two LGAs showed high level of resistance to Nalidixic acid. This could occur as a result of extra chromosomal mutation or mutation causing reduced drug accumulation (31). The isolated gram-negative bacteria also showed high level of resistance to Septrin and Ceporex. The gram-positive bacteria in both LGAs were resistant to erythromycin, amoxyl and ampiclox. This result is in line with the findings obtained by Gbojunbola *et al* in 2012 in which all the isolates were resistant to amoxyl (22) Resistance to amoxyl could be linked to the easy hydrolysis of the β -lactam ring by most bacteria as well as the frequent usage of the drug due to its low cost and availability. In the same vein, the least resistance was recorded against levofloxacin in most isolates. The emergence of drug resistance is one of the most serious problems in developing countries like Nigeria. This may occur as a result of misuse or overuse of antibiotics as well as poor infection prevention and control (12). The results showed that levofloxacin was the most potent and may be the drug of choice for treatment for infection associated with the isolated bacteria.

Two key factors that could potentiate the transmission of bacteria within different environment include population density and lifestyle. Port Harcourt metropolis consist of Obio/Akpor and Port Harcourt City LGAs. The population density of Obio/Akpor is greater than that of Port Harcourt. Also, Obio/Akpor has a lot of suburban areas unlike Port Harcourt.

E. coli expressed two resistant genes CTX-M, and SHV. These genes are extended spectrum beta lactamase (ESBL) genes. *E. coli* is one of the major bacteria that produce ESBL. In previous studies by Overdeest *et al.*, 2011 & Veenemans *et al.*, 2014, showed that drug resistance among *E. coli* has increased globally as a result of ESBL production (32, 33). Awor *et al.*, 2020 noted that the presence of ESBL producing *E. coli* in some poultry farms in Abuja (34). The presence of these bacteria in vegetables salad might be as result of using the poultry excreta as manure in the vegetables farms. *Klebsiella sp* which is also a known ESBL producer only showed positive to CTX-M. Apart from *S. aureus* that was positive for the VanB gene, the other gram positive isolates didn't express any resistance genes. This result could mean that antibiotic resistance expressed by these isolates was not as a result of possessing resistance genes. *E.coli* isolated from both LGAs exhibited same resistance genes (CTX-M and SHV) but were not resistant to the same drugs, it can be inferred that these genes might not be the only mechanism responsible for the resistance pattern observed.

Conclusion

The microbiological quality and safety of ready to eat salad sold in Port Harcourt metropolis seems to be a challenge despite the advances in technology and the awareness that has been created. The results obtained from this study exposes the poor microbiological quality of these salads which could result to health hazard.

Conflict of Interest

The authors declared no conflict of interest.

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