PREVALENCE AND RESISTIVITY OF GRAM-POSITIVE BACTERIA IN MINIMALLY PROCESSED SHELLFISH AND HARVEST WATER IN AKWA IBOM STATE, NIGERIA

www.akwapolyjournal.org
P-ISSN: 2536-6733 | E-ISSN: 2616-0773

Supported by: tetfund TERTIARY EDUCATION TRUST FUND
TETFund/DESS / POLY / IKOT-OSURUA/ ARJ /1-2)

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ABSTRACT

The prevalence and resistivity of Gram-positive bacteria in shellfish and harvest waters portends for food safety and improved regulatory compliance. We evaluated 180 shellfish samples and 30 water samples from three major harvest sites (Itu, Mbo, and Ibeno L.G.A) in Akwa Ibom State, Nigeria. The prevalence and concentrations of Gram-positive bacteria, specifically *Staphylococcus aureus*, *Micrococcus* sp, *Streptococcus* sp, and *Bacillus cereus*, were determined using standard microbiological methods. Results show there was a significant difference observed at 5 % ($p < .05$) in the heterotrophic count between the dry and rainy seasons, sample location, and bacterial colonization in water samples. However, the bacterial load in this study was above the stipulated range of $<10^4$ cfu/g by EU for *Bacillus* sp and *Micrococcus* sp in shellfish, while the bacterial load in some of the water

samples was also above the drinking and recreational water standard of EU. *Staphylococcus aureus* had the highest percentage occurrence of (35.71 %), followed by *Bacillus cereus* (28.57 %), *Streptococcus* sp. (20.41 %), and the least was *Micrococcus* sp. (15.31, %). The antibacterial susceptibility profiles of the isolates showed that *Bacillus cereus* had a resistance profile of 90 %, followed by *Streptococcus* sp (4 %), while *Staphylococcus aureus* (80 %) and *Micrococcus* sp (60 %) were susceptible to the antibiotics tested. These findings highlight the need for improved hygiene practices and regular monitoring of shellfish and harvest waters to reduce the risk of foodborne illnesses and antibiotic resistance transferred to humans.

Keywords: Shellfish, Gram-positive bacteria, harvest waters, food-borne illness, hygiene

INTRODUCTION

The consumption of various forms of aquatic or sea-life creatures (shellfish), a popular delicacy in Nigeria and the world at large, has been associated with a higher risk of food borne illnesses. Shellfish include Periwinkles, Crabs, Oysters, Scallops, Mussel, Clams, Shrimps, Crayfish, Sea Urchins, Sea Stars and Sea Cucumber. They can be processed for consumption by humans (1 Kin-Kabari *et al.,* 2017). The cultivation of these sea-life creatures, harvest, and marketing can serve as a veritable means of earning, vocation and food. From several reports, most of the areas where these shellfishes are harvested and processed are constantly faced with the challenges of faecal contamination from different sources which include wildlife within the catchment areas, livestock, pets and humans (2 Vincent-Hubert *et al.,* 2021). Shellfish are filter feeders implying that, they accumulate and concentrate pathogens as well as toxic chemicals from surrounding waters. Minimally processed shellfish, in particular, pose a significant threat due to their potential contamination with pathogenic microorganisms. Gram-positive bacteria, such as *Staphylococcus aureus, Bacillus*

cereus, and Clostridium pefringens, are among the most common causes of food poisoning associated with shellfish consumption.

To this end, consumers of raw or minimally processed shellfish products are exposed to harmful bacterial and viral pathogens. These sea-life creatures are a very important part of the food industry in Nigeria, with Akwa Ibom State as a major producer (3 Udoekong *et al*., 2021). Undoubtedly, when these shellfishes are cultivated and harvested from waters infested with pathogens, they become agents for pathogens-causing food borne infections when consumed raw or minimally processed. Furthermore, the misuse and overuse of antibiotics in aquaculture have contributed significantly to the rise of antibiotic-resistant bacteria, thereby complicating treatment and control efforts.

This study aims to investigate the prevalence and resistivity of Gram-positive bacteria in minimally processed shellfish and harvest water in Nigeria. By examining the types and levels of bacterial contamination, as well as the antimicrobial resistance profiles of isolated bacteria, this research seeks to provide valuable insights into the food safety risks
associated with shellfish consumption in Nigeria.

Materials and Methods:

Study Area

The research was conducted within some strategic coastal areas in Akwa Ibom State. These include Ibeno, Itu and Mbo Local Governement Areas all in the South-South region of the country.

Figure 1: Map Showing the Sampling Sites at Itu, Mbo, and Ibeno Local Government areas in Akwa Ibom State.

Sample Size:

For this study, the sample size was determined using Thrusfield's method (2007) using the equation (1) below.

$$n = \frac{1.96^2 \times Pexp\ (1-Pexp)}{d^2} \quad \text{……..…….(1)}$$

Where,

n = the required sample size.

t = the confidence level at 95 %

Pexp = expected frequency, and

d = the desired absolute precision.

Given an expected prevalence of 30.4 % (4 Adebayo-Tayo *et al*., 2012) and working with a precise precision of .05, the sample size n= 200

Sample Collection:

One hundred and eighty (180) samples of shellfish [oysters (*Crassostrea tulipa*), golden apple snail (*Pomacea canaliculata*), and clams (*Mercenaria mercenaria*] and thirty (30) samples of water were obtained for this study. The different samples of shellfish were obtained from three major harvest sites in Ibeno, Itu and Mbo Local Government Areas in Akwa Ibom State, Nigeria. In Nigeria, there are usually two distinct weather conditions: rainy (February to May) and dry (June to September) during the year. The grab sampler was used to obtain water samples from the different sites. The samples of water were collected weekly, early morning and evening during the dry season while mornings and after rainfalls during the rainy season. The samples were placed in ice-packed coolers (4^{o}C) and conveyed to the Science laboratory of the Akwa Ibom State Polytechnic, Osurua, Nigeria, for bacteriological investigations within six hours of collecting.

Sample Preparation:

The obtained Shellfish were washed individually and scrubbed using a sponge in a sterile water solution before rinsing in 70% Ethanol to remove external dirt and debris (3 Udoekong *et al*., 2021). The shellfish samples were shucked using a sterilized knife to cut-off the soft flesh. Each piece of flesh was separated into intestines and gills, as reported by (3 Udoekong *et al*., 2021). Five (5) g of the shellfish (dried and fresh) were homogenized using Stomacher R 400 Circulator, Seward Ltd. UK, soaked in 45 ml sterile physiological saline for five minutes. Samples were serially diluted in ten-folds and then spread-plated in duplicates onto nutrient agar plates (Difco Lab) for total heterotrophic bacterial counts (THBC). Bacterial growth was observed after incubating the samples for 24 hours at 37°C. To obtain pure cultures, the colonies that developed during this incubation period were counted and sub-cultured on new nutrient agar plates (5 Cheesbrough, 2006).

Pre-enrichment, Isolation and Presumptive Bacterial Identification:

A 500 ml Erlenmeyer flask filled with 225 ml of lactose broth (Oxoid, UK) was filled with a 25 g homogenate of each shellfish, which was then pre-enriched for 24 hours at 37°C in an incubator (Kemi, India) [5 ISO 6579]. Using a sterile pipette, 0.1 and 1 ml of the pre-enrichment

were transferred in duplicates into prepared McConkey Agar, Mannitol Salt Agar, and Tryptic Soy Agar plates. For 24 hours, the plates were incubated at 37^{0}C. The streak

plating method was adopted for the purification of discrete bacterial colonies placed on nutrient agar and incubated for 24 hours at 37^0C. Following sub-culturing as pure cultures, the colonies were kept on nutrient agar slants and then refrigerated at 4^0C for additional examination. The techniques described in Cheesbrough (2006) were used to presumptively characterize the purified colonies.

The cultural, morphological, Gram reaction, motility, and common biochemical tests used in this characterization included catalase, coagulase, urease, endospore, oxidase, nitrate reduction, and carbohydrate utilization tests. The bacterial isolates were validated by sequencing the isolates' 16S ribosomal RNA and finding a 100% match with the NCBI gene bank.

Antibiotic Susceptibility Test:

The bacterial isolates' susceptibility to antimicrobial agents was assessed using the Kirby-Bauer disc diffusion method [5; 7; 8 Cheesbrough, Fallah; Wilker] adhering to the protocols established by the Clinical and Laboratory Standards Institute. This study used discs that were procured commercially. They were cloxacillin (5 µg), vancomycin (30 µg), gentamycin (10 µg), erythromycin (15 µg), clindamycin (2 µg), amoxicillin (10 µg), oxacillin (1 µg), nafcillin (1µg), chloramphenicol (30 µg), and Tetracycline (30 µg). Using a sterile inoculating loop, the culture from the stock was transferred into tubes with 2.5 ml of sterile normal saline to create a bacterial lawn, which was then cultured for six hours at 37ºC. Barium chloride was used to compare the suspension's density (0.5 McFarland Turbidity Standard; 1.0 x108 cfu/µL). 0.1 ml of the inoculum was poured onto Meuller-Hinton agar plates that had been produced and dried using the spread plate procedure. The surplus inoculum was carefully removed with sterile Pasteur pipettes, and the antibiotic discs were meticulously positioned on the surface of the inoculated medium at uniform intervals using sterile forceps. For eighteen hours, the seeded plates were inverted and incubated at 37ºC. The inhibitory zones' diameters were measured and interpreted following the US Clinical and Laboratory Standard Institute [5;9 Cheesbrough ; Mafu].

Minimum Inhibitory Concentration of the Bacterial Isolates:

The bacterial isolates' Minimum Inhibitory Concentration (MIC) was determined using the [10] Andrews *et al*. (2001) technique. For this research, the E-test strip was used. Using a sterile inoculating loop, the culture from the stock was transferred into tubes with 2.5 ml of sterile normal saline to create a bacterial lawn, which was then incubated

for four hours at 37 ^{o}C. Barium chloride was used to compare the suspension's density (0.5 McFarland Turbidity Standard; 1.0 x10^{8} cfu/µL). After being dipped into the inoculum, a sterile swab stick was twisted, brushing the tube's edge to remove any extra fluid, and streaked across the prepared and dried Agar Mueller-Hinton plates. The excess moisture was allowed to absorb before placing the E-test strip gently onto the seeded agar to ensure contact using sterile forceps and ensuring the antibiotic gradient faces upwards. Inverted incubation was used for the seeded plates at 37 ^{o}C for 24 hours. After the incubation, the elliptical areas where inhibition occurs around the E-test strips were examined and evaluated following the US Clinical and Laboratory Standard Institute. A quality control strain confirmed the E-test strip potency (*E. coli* ATCC 25922).

Molecular Analysis:

16S rRNA amplification

The amplification of the 16S rRNA region of the isolates' rRNA genes was carried out using the 27F: 5^{1}-AGAGTTTGATCMTGGCTCAG-3^{1} and 1492 R: 5^{1}-CGGTTACCTTGTTACGACTT - 3^{1} primers on an Applied Biosystems AB1 9700 heat cycler for 35 cycles with a final volume of 50 µl. Inqaba, South Africa, provided the X2 Dream Taq Master Mix (Taq polymerase, DNTPs, MgCl), which was used for the amplification. The primer concentration was 0.4 M, and the extracted DNA served as the template. Initial denaturation at 95 ^{o}C for 5 minutes, subsequent denaturation at 95 ^{o}C for 30 seconds, annealing at 52 ^{o}C for 30 seconds, extension at 72 ^{o}C for 30 seconds for 35 cycles, and a final extension at 72 ^{o}C for 5 minutes were the conditions necessary for the PCR amplification. A UV trans-illuminator was used to visualize the bands after the amplification product was applied to a 1.0% agarose gel, and run at 120 V for 20 minutes.

DNA extraction

Extraction was performed using the ZR fungal/bacterial DNA mini prep extraction kit supplied by Inqaba, South Africa. A heavy growth of the pure culture of the suspected isolates was suspended in 200 microliters of isotonic buffer within ZR Bashing Bead Lysis tubes. Then, 750 microliters of lysis solution was added to each tube. The tubes were secured in a bead beater equipped with a 2 ml tube holder assembly and processed

at maximum speed for 5 minutes. Afterward, the ZR Bashing Bead Lysis tubes were centrifuged at 10,000 x g for 1 minute.

In a collecting tube, 400 microlitres of the supernatant were moved to an orange-top Zymo-Spin IV spin filter, and they were centrifuged for one minute at 7000 xg. The filtrate in the collection tubes was mixed with 1,200 microlitres of fungal/bacterial DNA binding buffer to reach a final volume of 1600 microlitres. Eight hundred microlitres were then moved to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000xg for one minute; the flow through was then disposed of from the collection tube. After being moved to the same Zymo-spin, the remaining volume was spun. The Zymo-spin IIC was filled with 200 microlitres of DNA Pre-Was buffer in a fresh collection tube. It was then spun at 10,000xg for one minute. Next, 500 microlitres of fungal/bacterial DNA Wash Buffer was added, and it was centrifuged at 10,000xg for one minute.

To elute the DNA, the Zymo-spin IIC column was moved to a sterile 1.5 microlitre centrifuge tube, along with 100 microlitres of DNA elution buffer. The column matrix was then centrifuged at 10,000xg microlitre for 30 seconds. After that, the ultra-pure DNA was kept at -20 degrees for further reactions.

DNA quantification

The Nanodrop 1000 spectrophotometer was used to measure the amount of isolated genomic DNA. The Nanodrop icon was double-clicked to activate the equipment's software. Two microlitres of sterile distilled water were used to initialise the apparatus, and regular saline was used to blank it. After loading two microlitres of the extracted DNA onto the lower pedestal, the upper pedestal was lowered so that it came into contact with the lower pedestal's extracted DNA. By selecting the "measure" button, the DNA concentration was determined.

Amplification of resistance genes

a) Amplification of tetracycline (*tet*) resistance genes

The isolates' ***tet*** genes were amplified using the tetF***:*** 5´-TTGGCATTCTGCATTCACTC -3´ and ***tet*R*:*** 5´- GTATAGCTTGCCGGAAGTCG -3´ primers for 35 cycles at a final volume of 30 microlitres using an ABI 9700 Applied Biosystems heat cycler. The PCR mix contained 50ng of the extracted DNA as a

template, primers at a concentration of 0.4uM, and the X2 Dream Taq Master mix (Taq polymerase, DNTPs, MgCl) provided by Inqaba, South Africa. The following were the PCR conditions: Initial denaturation: 5 minutes at 95ºC; subsequent denaturation: 30 seconds at 95ºC; annealing: 30 seconds at 60ºC; extension: 72ºC for 30 seconds for 35 cycles; and final extension: 5 minutes at 72ºC. A blue transilluminator was used to view the result after it had been resolved on a 1% agarose gel at 130V for 25 minutes.

b) Amplification of erythromycin (*erm*) resistance genes

We successfully amplified the ***erm*** genes from the isolates using the highly specific primers, ***ermF***: 5' AGAAGGTTATAATGAAACAGA -3' and *ermR*: 5' GGCATGACATAAACCTTCAT -3', on a ABI 9700 Applied Biosystems thermal cycler. This crucial process was carried out in a final volume of 30 microliters for an optimized 35 cycles. The PCR mix was meticulously prepared with the X2 Dream Taq Master mix from Inqaba, South Africa, which includes Taq polymerase, DNTPs, and MgCl. We employed the primers at a concentration of 0.4 µM and utilized 50 ng of the extracted DNA as our template.

The PCR conditions were rigorously controlled: starting with an initial denaturation at 95ºC for 5 minutes, followed by denaturation at 95ºC for 30 seconds, annealing at 60ºC for 30 seconds, and extending at 72ºC for 30 seconds over the course of 35 cycles. Finally, we ensured an extended finalization at 72ºC for 5 minutes. The resultant product was precisely resolved on a 1% agarose gel, run at 130V for 25 minutes, and effectively visualized using a blue transilluminator.

Statistical Analysis:

Statistical analysis was performed using analysis of variance (ANOVA) and Duncan's test. A P-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

Results

Results for the bacteriology and resistance patterns of different samples of shellfish and its harvest waters obtained from three landing sites in Akwa Ibom State, Nigeria showed diverse outcomes. Figure 1 shows that there was no significant difference observed in the results. the average number of positive cultures in the different sampling points ($p>.05$). The mean quantity of cultures that are good in the different types of shellfish was significantly higher than that obtained in harvest water ($p<.05$) while in the different types of shellfish, there was no significant difference in the

average number of positive culture (p>.05) (Figure 2). Meanwhile, the average bacterial colonization in Ibeno was significantly less than that obtained in Itu and Mbo (p<.05); while between Itu and Mbo, there was no significant difference in their bacterial colonization (p>.05) (Figure 3). The result for the average bacterial colonization in the dry season was significantly higher than wet season (p<.05) (Figure 4). The result from Figure 5 shows that heterotrophic counts for bacteria in Ibeno were significantly greater than that in Itu and Mbo (p<.05) while that of Itu was significantly lower compared to Mbo (p<.05). The heterotrophic counts for bacteria in Clams was significantly higher than in Oyster and golden apple snail while that of Oyster was significantly greater than that found in golden apple snail (p<.05) (Figure 6). Figure 7 shows that heterotrophic counts for bacteria was not significantly different between dried and fresh fish types (p>.05) though slightly higher in dried than fresh. For the seasonal variations, the heterotrophic counts for bacteria was significantly higher in the dry season than raining season (p<.05) (Figure 8).

Bacterial species identified using 16S rRNA gene sequences from the shellfish samples were *Staphylococcus aureus*, *Micrococcus* sp., *Streptococcus* sp., and *Bacillus cereus* (Table 1).
From the study, all the bacterial isolates were recovered from the shellfish and water samples except *Streptococcus* sp which was not isolated from the water samples. *Staphylococcus aureus* had the highest percentage occurrence from fresh (40.00%) and dry (35.71%) shellfish samples. *Micrococcus* sp. isolate was the least (22.86%) observed from the harvest water sample
(Table 2).

The patterns of antimicrobial susceptibility of four (4) Gram-positive bacterial isolates were determined using 10 selected antimicrobial agents (cloxacillin, vancomycin, gentamycin, erythromycin, clindamycin, amoxicillin, oxacillin, and nafcillin) and revealed diverse resistance profiles. These profiles are summarized in Table 3.

Bacillus cereus showed 90 % resistance to the tested antibiotics except for amoxicillin, followed by *Streptococcus* sp. with 60 % resistance to 6 antibiotics except for vancomycin, gentamycin, amoxicillin, and chloramphenicol. The isolates of bacterial Minimum Inhibitory Concentration (MIC) in this study revealed that most results were

below the breakpoint of the antibiotics tested, thus signifying susceptibility according to the guidelines by CCLS (Table 4).

The isolates examined in this research demonstrated the antibiotics resistance genes, *erm*, *tet* and *cat* (Table 5).

Staphylococcus aureus expressed the antibiotics resistance genes *tet* (resistance to tetracycline) and *erm* (resistance to erythromycin). *Micrococcus* sp. expressed the *tet*, *erm*, and *cat* (resistance to tetracycline, erythromycin and chloramphenicol) genes. *Streptococcus* sp. expressed the *erm*, *tet* genes (resistance to erythromycin and tetracycline). *Bacillus cereus* expressed the *tet*, *cat*, and *erm* (resistance to tetracycline, chlororamphenicol and erythromycin antibiotics).

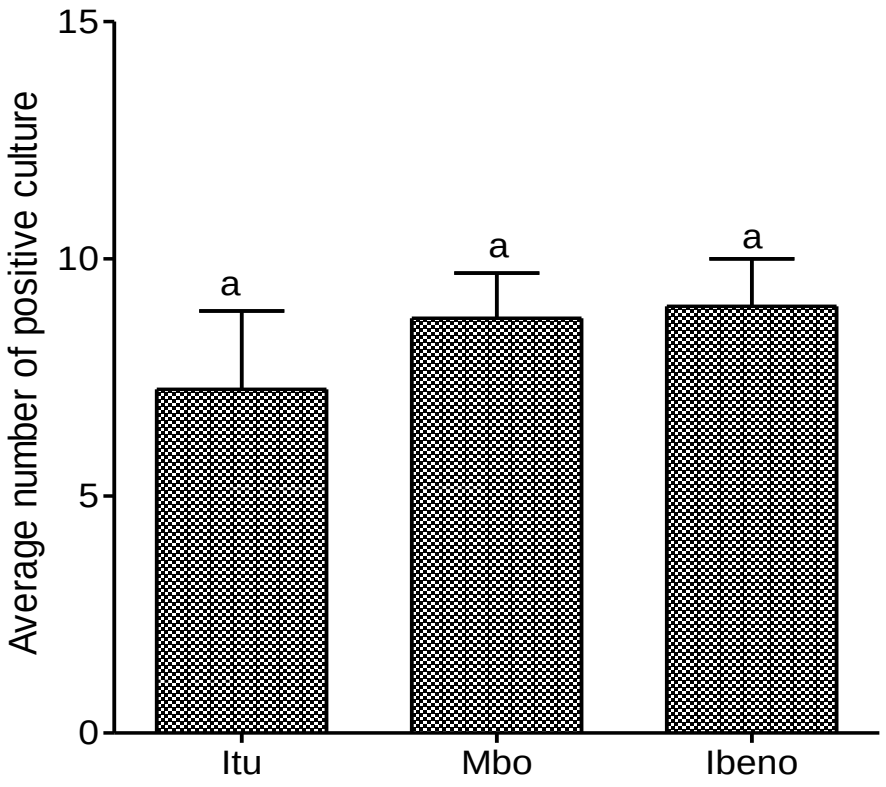

FIGURE 1: Average Number of Positive Cultures By Sampling Points

Values reported as mean±SEM, similar superscript letters means not significantly different at 5% (p>.05) while different superscript letters means significantly different at 5% (p<.05).

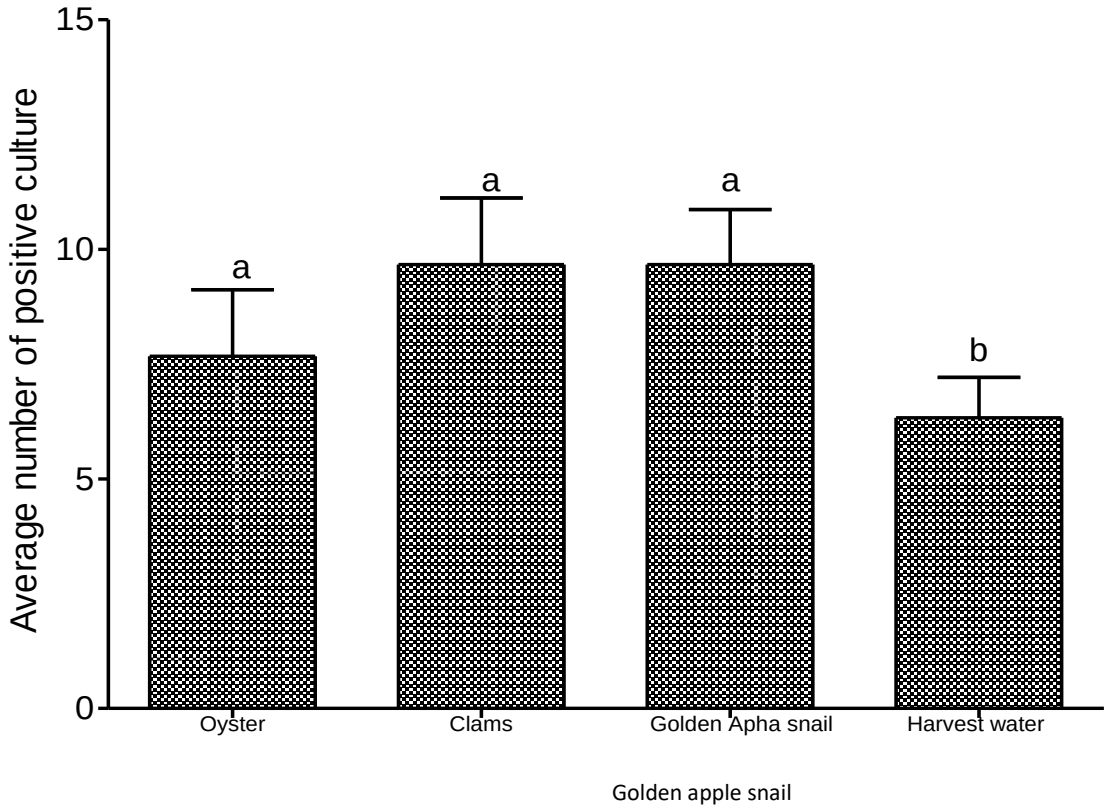

FIGURE 2: Average Number of P(Types

Values reported as mean±SEM, similar superscript letters means not significantly different at 5% (p>.05) while different superscript letters means significantly different at 5% (p<.05).

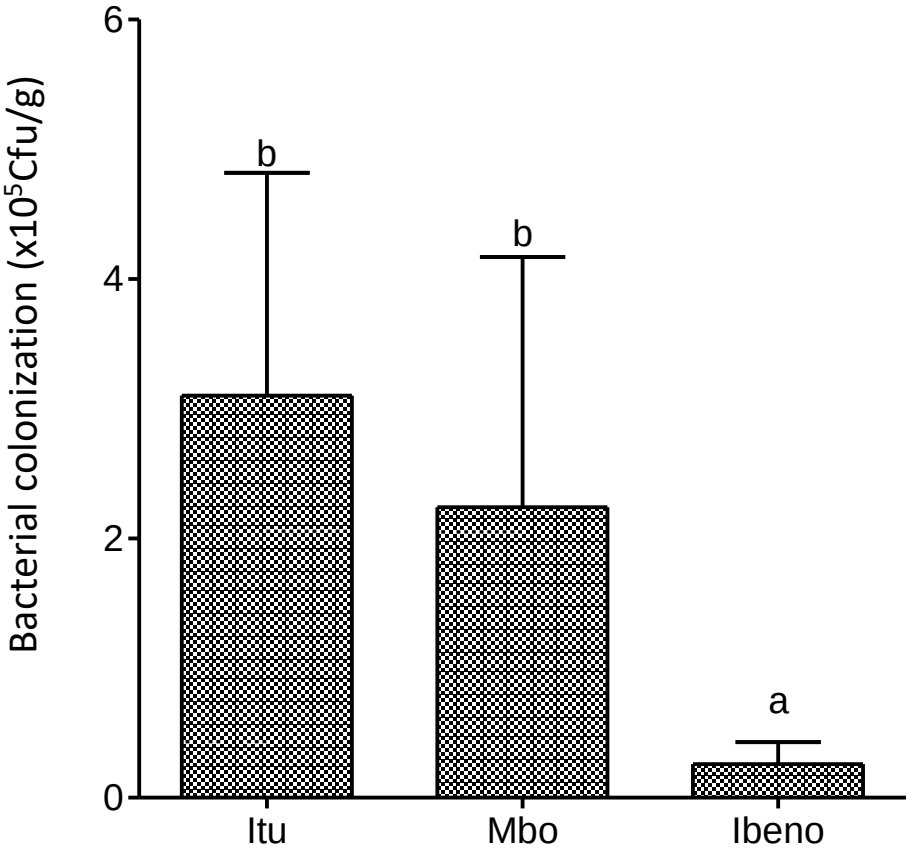

FIGURE 3: The Bacterial Colonization by sampling points

Values reported as mean±SEM, similar superscript letters means not significantly different at 5% (p>.05) while different superscript letters means significantly different at 5% (p<.05).

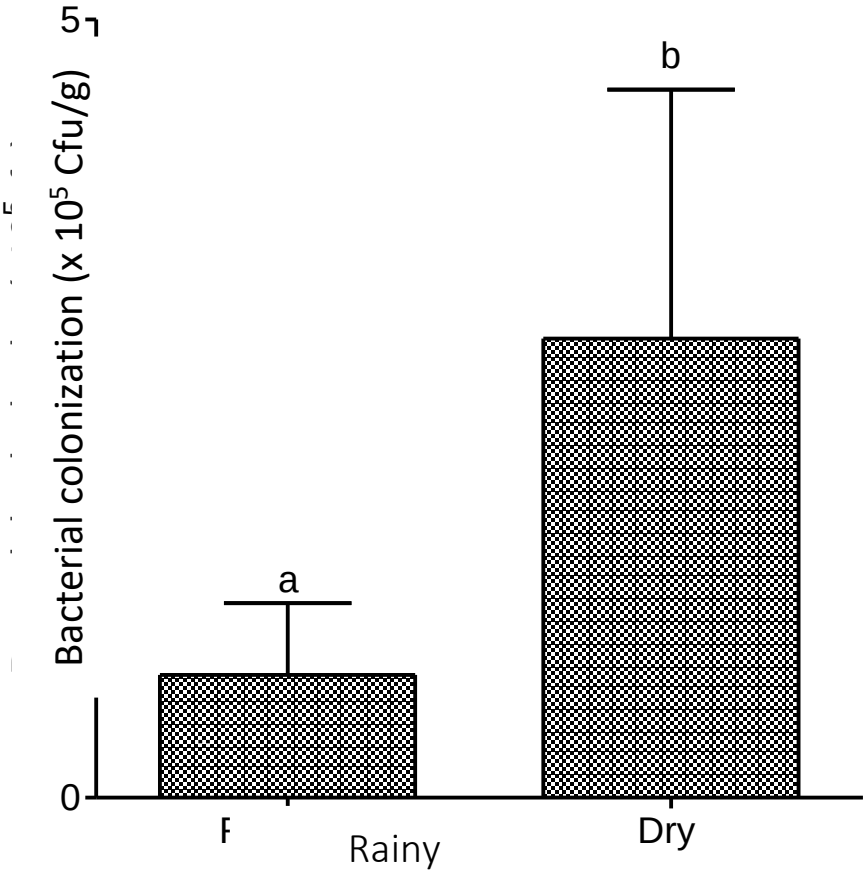

FIGURE 4: Bacterial Colonization Between seasons

Values reported as mean±SEM, similar superscript letters means not significantly different at 5% (p>.05) while different superscript letters means significantly different at 5% (p<.05).

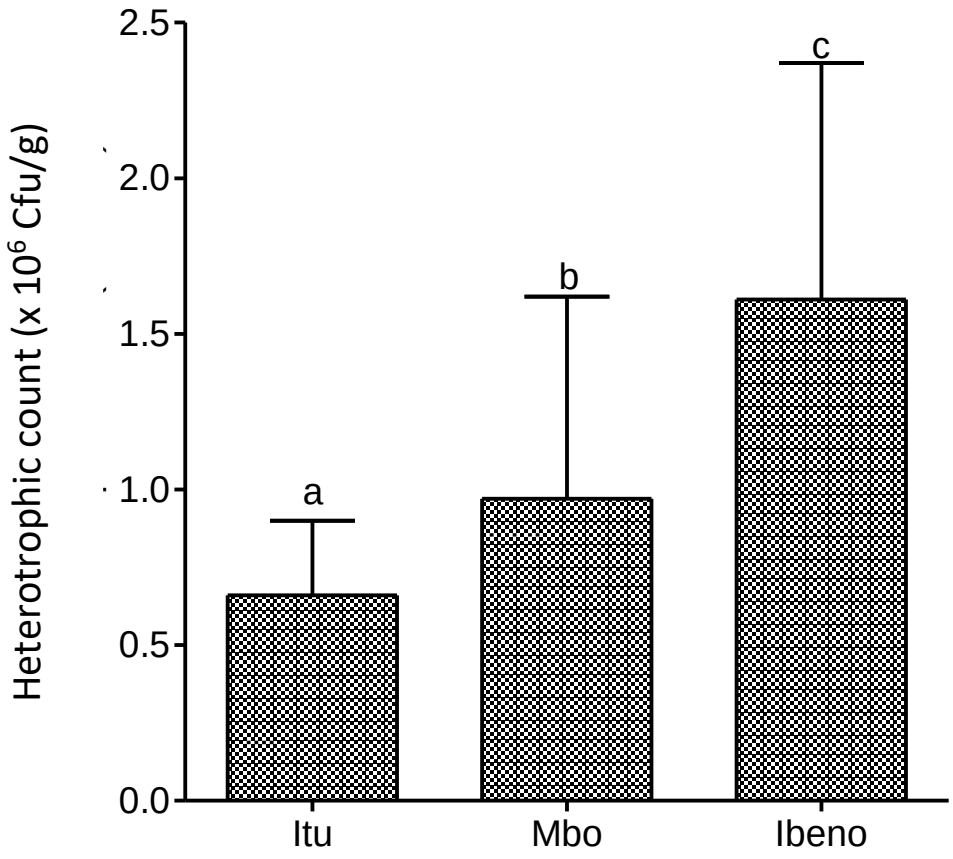

FIGURE 5: Heterotrophic Counts between the Sampling Points

Values reported as mean±SEM, similar superscript letters means not significantly different at 5% (p>.05) while different superscript letters means significantly different at 5% (p<.05).

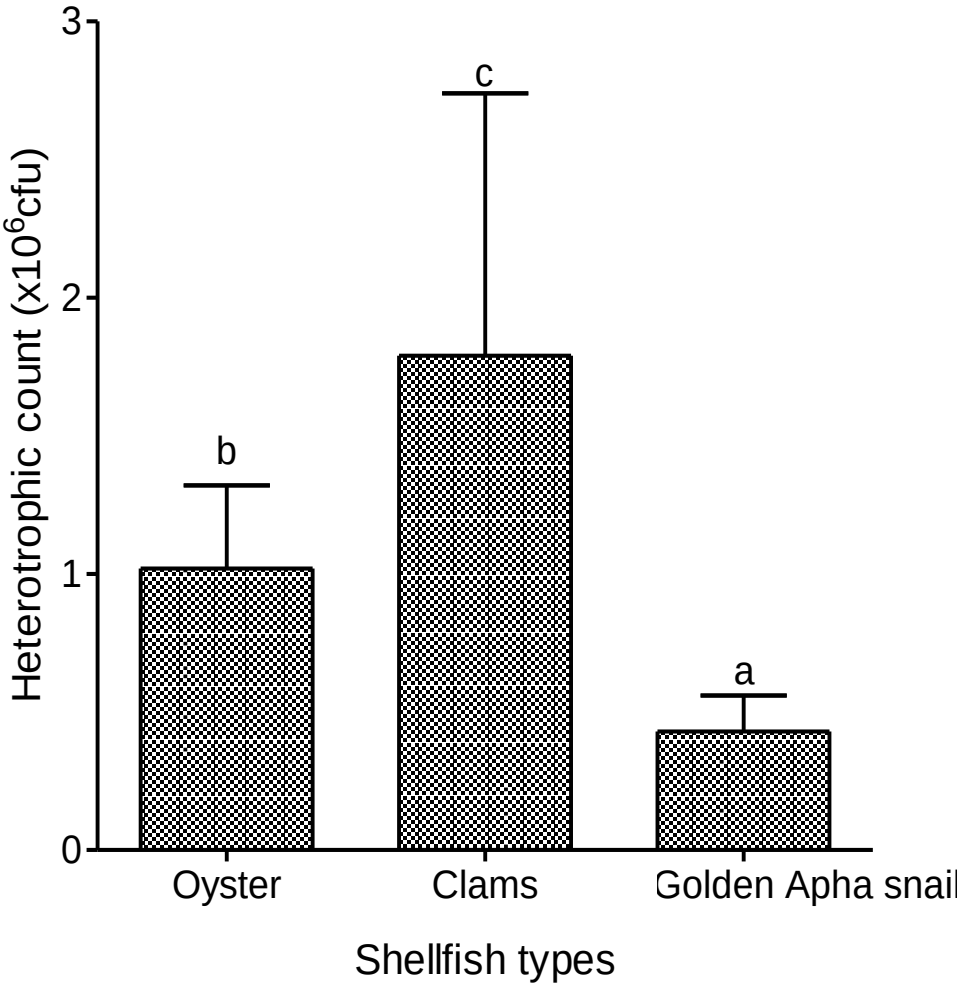

FIGURE 6: Total Heterotrophic Count Between the Shellfish Types

Values reported as mean±SEM, similar superscript letters means not significantly different at 5% (p>.05) while different superscript letters means significantly different at 5% (p<.05).

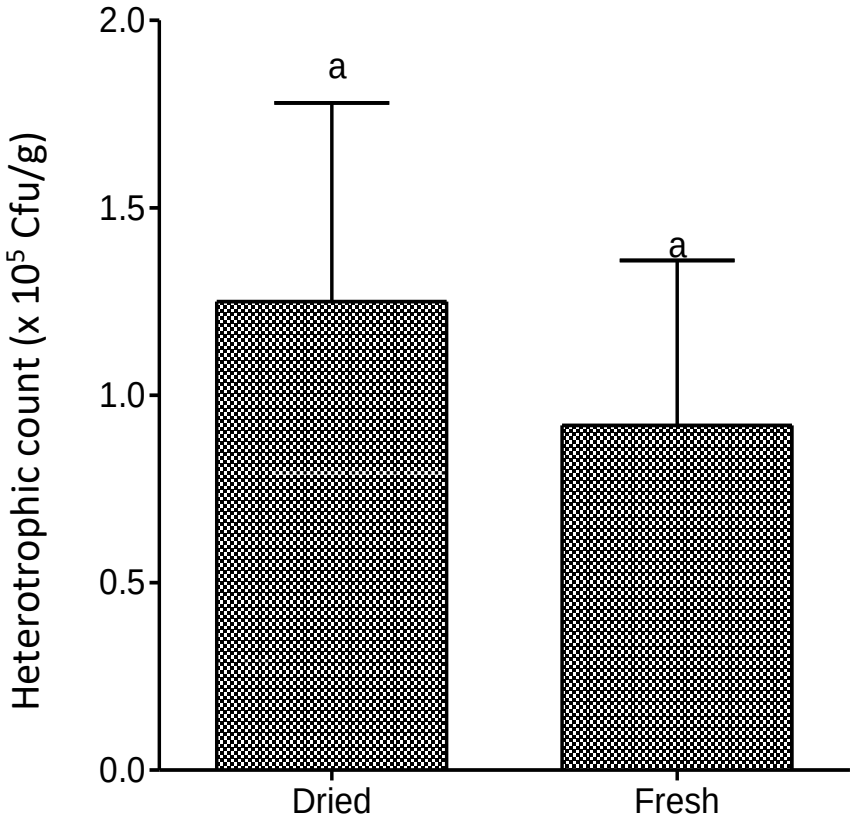

FIGURE 7: Total Heterotrophic Counts Between the Shellfish Forms

Values reported as mean±SEM, similar superscript letters means not significantly different at 5% (p>.05) while different superscript letters means significantly different at 5% (p<.05).

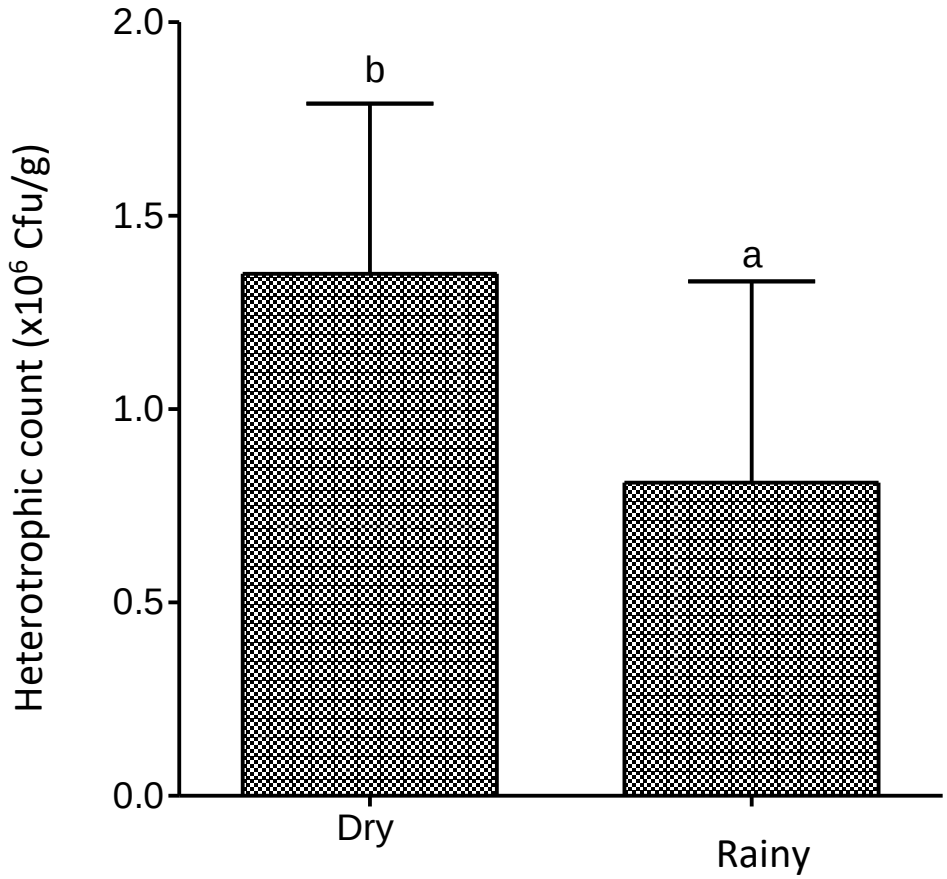

FIGURE 8: Total Heterotrophic count between the Seasons

Values reported as mean±SEM, similar superscript letters means not significantly different at 5% (p>.05) while different superscript letters means significantly different at 5% (p<.05).

Table 1: Identities of the bacterial isolates

Sample Code	Isolated Bacteria	Species
Ns 1	*Staphylococcus*	*aureus*
Ns 2	*Micrococcus* sp.	-
Ns 3	*Streptococcus* sp.	-
Ns 4	*Bacillus*	*cereus*

Table 2: Frequency and percentage occurrence of the bacterial isolates from the samples

	Shellfish		
Isolated Bacteria	**Frequency/% occurrence n=75 Fresh**	**Frequency/% occurrence n=98 Dried**	**Harvest Water n= 35**
Staphylococcus aureus	30 (40.00)	35 (35.71)	17 (48.57)
Micrococcus sp.	10 (13.33)	15 (15.31)	8 (22.86)
Streptococcus sp.	15 (20.00)	28(28.57)	-
Bacillus cereus	20 (26.67)	20 (20.41)	10 (28.57)
Total	**75**	**98**	**35**

Table 3: Resistivity profiles of the bacterial isolates from the samples

	Bacterial Isolates			
Antibiotics	***Staphylococcus aureus***	***Streptococcu***	***Bacillus cereus***	***Micrococcus* sp.**
Cloxacillin (5 µg)	S	R	R	I
Vancomycin (30 µg)	S	S	R	S
Gentamycin (10 µg)	S	I	R	S
Erythromycin (15 µg)	S	S	R	S
Clindamycin (2 µg)	I	I	R	S
Amoxicillin (10 µg)	S	S	R	I
Oxacillin (1 µg)	S	R	R	S
Nafcillin (1 µg)	S	R	R	S
Chloramphenicol (30 µg)	S	S	R	I
Tetracycline (30 µg)	I	R	I	R

Key: R (Resistance in mm), cloxacillin (5 µg) R≤13, gentamycin (10µg) R≤12, erythromycin (15µg) R≤14, clindamycin (2 µg) R≤15, amoxicillin (10 µg) R≤13, oxacillin (1 µg) R≤9, nafcillin (1 µg) R≤11, chloramphenicol (30 µg) R≤13, tetracycline (30 µg) R≤14

Table 4: Minimum Inhibitory Concentration (MIC) of the Bacterial Isolates

	Gram-Positive Antibiotics									
Isolated bacteria	**oxacillin (1 µg/ml)**	**cloxacillin (5 µg/ml)**	**amoxicillin (10 µg/ml)**	**vancomycin (30 µg/ml)**	**gentamycin (10 µg/ml)**	**erythromycin (15 µg/ml)**	**clindamycin (2 µg/ml)**	**nafcillin (1 µg/ml)**	**chloramphenicol (30 µg/ml)**	**tetracycline (30 µg/ml)**
Staphylococcus aureus	-	0.5	0.5	2	2	0.5	0.5	0.25	8	8
Streptococcus sp	-	-	2	1	-	0.25	1	2	8	16
Micrococcus sp	0.25	1	1	4	4	0.5	0.5	0.25	16	16
Bacillus cereus	-	-	2	-	16	8	4	2	32	16

Key: R **(**Resistance in mm**)** *Staphylococcus aureus* cloxacillin (5 µg/mL) R≥4, gentamycin (10 µg/mL) R ≥16, erythromycin (15 µg/mL) R ≥8, clindamycin (2 µg/mL) R ≥ 8, amoxicillin (10 µg/mL) R ≥ 4, vancomycin (30 µg/mL) R ≥ 16, oxacillin (1 µg/mL) R ≥ 9, nafcillin (1 µg/mL) R ≥ 4, chloramphenicol (30 µg/mL) R ≥ 64, tetracycline (30 µg/mL) R ≥ 32.

***Streptococcus* sp**. cloxacillin (5µg/mL) R≥2, gentamycin (10µg/mL) R≥4000, erythromycin (15µg/mL) R≥2, clindamycin (2µg/mL) R≥2, amoxicillin (10µg/mL) R≥2, vancomycin(30µg/mL) R≥8, oxacillin (1µg/mL) R≥2, nafcillin (1µg/mL) R≥2, chloramphenicol (30µg/mL) R≥64, tetracycline (30µg/mL) R≥16.

***Micrococcus* sp**. cloxacillin (5 µg/mL) R ≥ 4, gentamycin (10 µg/mL) R ≥ 32, erythromycin (15 µg/mL) R ≥ 8, clindamycin (2 µg/mL) R ≥ 8, amoxicillin (10 µg/mL) R ≥ 4, vancomycin (30 µg/mL) R ≥ 32, oxacillin (1 µg/mL) R ≥ 4, nafcillin (1 µg/mL) R ≥ 4, chloramphenicol (30 µg/mL) R ≥ 64, tetracycline (30 µg/mL) R ≥ 32.

***Bacillus* sp.** cloxacillin (5 µg/mL) R ≥ 4, gentamycin (10 µg/mL) R ≥ 32,

erythromycin (15 µg/mL) R ≥ 8, clindamycin (2 µg/mL) R ≥ 8, amoxicillin (10 µg/mL) R ≥ 4, vancomycin (30 µg/mL) R ≥ 32, oxacillin (1 µg/mL) R ≥ 4, nafcillin (1 µg/mL) R ≥ 4, chloramphenicol (30 µg/mL) R ≥ 64, tetracycline (30 µg/mL) R ≥ 32.

Table 5: Distribution of the resistance genes among the isolated bacteria from the samples

Sample Code	Isolated Bacteria	Antibiotics Genes
Ns 1	*Staphylococcus aureus*	*tet, erm*
Ns 2	*Micrococcus* sp	*tet, erm, cat*
Ns 3	*Streptococcus* sp	*tet, erm*
Ns 4	*Bacillus cereus*	*tet, erm, cat*

Discussions:

This study demonstrates the presence of potentially harmful Gram-positive bacteria in minimally processed shellfish and harvest waters in evaluated landing sites around Akwa Ibom State coastal cities. This study found high levels of heterotrophic bacteria in both shellfish and water samples analyzed. Notably, clams exhibited a higher bacterial count compared to the other shellfish examined. The elevated levels of heterotrophic bacteria in clams and oysters may be linked to their unique filter-feeding mechanisms, as well as their intricate and well-developed symbiotic relationships with microbes. [11]. These results highlight the need for increased public awareness about the risks of consuming contaminated seafood and drinking untreated water. In most parts of Nigeria (as in most coastal areas in Africa), shellfish like Clams, oysters, periwinkles, and similar seafood are commonly eaten as sources of dietary protein. as opined by [3]. These become potential vehicles for transmitting pathogenic microorganisms [1].

Many anthropogenic activities and rainfall, which contributes to soil surface run-off, are believed to influence total heterotrophic bacteria counts in these regions [12]. However, our seasonal distribution analysis indicates that mean heterotrophic bacteria counts were higher during the dry season compared to the rainy season. This finding

aligns with earlier research by Silva-Neta et al. [13], which reported increased bacterial concentrations in certain seafood at the end of the dry season. Bacteria in aquatic ecosystems can be indigenous or exogenous, and they can be either persistent or transient, depending on their origin from animals, plants, or soil surfaces. [14].

Additionally, seafood pollution can happen at various stages. during processing and transportation [15]. The degree of pollution in seafood. mainly depends on the pollution levels in the environment and during processing [16]. We noted that 95% of the bacterial load readings surpassed the acceptable contamination thresholds for shellfish as defined by the International Commission on Microbiological Specifications for Food (ICMSF) and the Centre for Food Safety and Applied Nutrition (CFSAN) of the US Food and Drug Administration [16]. The most secure choice for consumers in these regions is to stop consuming minimally processed seafood and properly cook them before consumption.

Furthermore, through media and awareness programs, governments and agencies should underscore the need for improved sanitation and hygiene practices in shellfish harvest and processing. The release of effluents like untreated sewage and antimicrobials into wastewater pollutes water bodies and affects shellfish sourced from these areas. This situation possibly plays a role in the growing occurrence of antibiotic-resistant bacteria and genes in aquatic environments, which poses a major concern for environmental health. The identification of antibiotic resistance in pathogens found in seafood underscores human activities' significant and detrimental impact on aquatic ecosystems. This affects the microbial community and poses significant health risks to humans, underscoring the urgent need for more effective stewardship of antibiotics and implementing measures to mitigate the spread of antibiotic resistance [14].

Antimicrobial resistance (AMR) is a re-emerging health issue [17]. Efforts to elucidate associated problems are underscored by the importance of routine antimicrobial susceptibility testing [18]. The isolates in this study expressed notable virulence markers such as the antibiotic resistance genes (*tet*, *erm*) by *Staphylococcus aureus* in shellfish and the harvested water [18; 19]. In this study, *Micrococcus* sp is an indicator organism for water quality assessment and gains entry to shellfish and water through the indiscriminate disposal of human sewage, wastewater, recreational water, stormwater run-off, and industrial effluent. *Streptococcus* sp in this study signifies fecal

contamination of the shellfish and harvest water through recreational activities, water runoff, industrial effluent, human sewage, and untreated wastewater disposal into the water bodies. The bacterium possess the resistance genes *tet* (resistance to tetracycline) and *erm* (for erythromycin antibiotic). *Bacillus cereus* also indicates soil and fecal contamination. The bacterium gains access to shellfish and harvest water through human sewage disposal, wastewater, industrial effluent, and soil sediment. *Bacillus cereus* also expressed the *tet*(resistance to tetracycline), *erm*(for erythromycin antibiotic), and *cat* (chloramphenicol resistant gene).

Conclusion:

Our study's findings highlight the urgent need to address the health risks of consuming minimally processed shellfish, especially those harvested from polluted ecosystems. To mitigate the spread of foodborne illnesses and protect public health, regulatory agencies and stakeholders must collaborate on implementing enhanced hygiene practices and monitoring protocols, such as regular testing of shellfish for pathogens, improved sanitation in harvesting and processing facilities, and greater consumer awareness of food safety risks.

Our findings indicate that the bacteria levels in vended shellfish from Itu, Mbo, and Ibeno beaches in Akwa Ibom State exceed the acceptable limits for consumable shellfish products. This raises serious concerns about the safety of these products and underscores the need for improved hygiene practices in shellfish harvesting, handling, and processing to prevent the spread of foodborne illnesses. The analysis of antimicrobial susceptibility testing revealed that Gram-positive bacterial isolates from shellfish displayed significant resistance rates to various antimicrobial agents. Bacillus cereus, in particular, exhibited considerable resistance to most of the tested antimicrobial agents, with the exception of tetracycline. Additionally, the isolated Streptococcus species demonstrated resistance to tetracycline, nafcillin, oxacillin, and cloxacillin. In summary, Gram-positive bacteria (GPB) present in shellfish could increase the risk of spreading multi-drug resistance, which poses a serious public health threat.

Recommendations

Based on the findings from this study, the following are recommended;

1. Regular monitoring of shellfish and harvest waters for Gram-positive bacteria.

2. Implementation of improved hygiene practices during shellfish handling and processing.
3. Public education campaigns to promote safe seafood consumption practices.
4. Future studies should consider expanding the sample size and scope to include other types of seafood and harvest sites.

COMPETING INTERESTS

The authors have asserted that no conflicts of interest exist.

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