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Microbiological Assessment and Shelf Life Study of Periwinkle Preserved by Smoking, Polythene and Vacuum-Packing

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Abstract: *In this study, freshly harvested periwinkle (Tympanotosus fuscatus) was smoked, then divided into three portions - polythene packaged smoked periwinkle (PPSP), vacuum packaged smoked periwinkle (VPSP) and non-packaged smoked periwinkle (NPSP) and were stored at ambient temperature (28±2 ^{o}C). At intervals, total bacterial and fungal count of these samples were monitored using Standard microbiological methods for 2-16 weeks. Further identification of the isolates involved the use of Molecular methods. A total of nine bacterial species were isolated from freshly harvested periwinkle (FHP) while five, three and two bacterial species were isolated from PPSP, VPSP and NPSP, respectively. The isolates were Vibrio sp., Staphylococcus epidermidis strain 11069, Escherichia coli strain M11957, Klebsiella pneumoniae strain OG039, Providencia sneebia, Pseudomonas aeruginosa strain OG003, Chryseobacterium aquifrigidense strain C3, and Bacillus flexus Mj-2. Also encountered in the samples were fungal isolates namely Aspergillus niger strain 5-F34 Penicillium citrinum strain 33 and Nectriaceae sp. clone SF_98. The predominant bacteria in FHP and PPSP is B. flexus while that of VPSP and NPSP involved S. epidermidis. Sample PPSP and NPSP have a short shelf life (< 2 Weeks) whereas sample VPSP has a longer shelf life (12 Weeks) with reference to the total plate count of 5 log_{10}CFU/g (max.) for shellfish recommended by International Commission on Microbiological Specifications for Food (ICMSF) and maximum total fungal count less than 10^4 log_{10}CFU/g. Therefore, vacuum packaging is recommended for extending the shelf life of smoked periwinkle.*

Keywords: *Periwinkle, shelf life, vacuum packing, polythene packing, smoking*

1. INTRODUCTION

Periwinkles are gastropods that belong to the Phylum Mollusca. It is a shellfish that has a soft body. Three predominant genera of periwinkles are *Tympanotosus*, *Pachymelania* and *Merceneria*. In Nigeria, especially Niger Delta regions, *Tympanotosus fuscatus* and *Pachymelania aurita* are very common (Jimmy and Okonkwo, 2016; Inyang *et al*., 2018). *Pachymelania aurita* is characterized by a sharp spine. The sharpness of the spine is dependent on the age of the specie. *Pachymelania aurita* has a broader aperture than *Tympanotosus fuscatus* which has granular, turreted and spiny shells with tapering ends (Aigberua and Izah, 2018; Abiaobo and Asuquo, 2020). Periwinkles are usually found in the sea, mostly in the brackish and littoral regions. It is a desirable seafood because it is a cheap source of protein, vitamins and minerals. Periwinkle is well appreciated because of its low cholesterol, fats and carbohydrate content (Archibong *et al.*, 2014; Nrior *et al*. 2017; Ngozi *et al.*, 2020).

In recent years, there has been an increase in demand for periwinkle due to its culinary value among the people of Niger Delta. Despite anthropogenic pollution that characterize the region in the past few decades, periwinkles have been able to adapt to the challenging environment and remains available for harvest all year round (Adebayo-tayo *et al.*, 2008). However, human development such as land reclamation to erect houses, crop farming, and industrialization is gradually reducing the habitat available for periwinkle (Abiaobo and Asuquo, 2020). Enriched microbial community of water bodies in the Niger Delta is attributed to the constant release of untreated inorganic and organic pollutants from human, animal, domestic and industrial wastes. Bulk of the industrial wastes generated in that region is attributed to crude oil related activities. Over a period, periwinkle and other sea foods inhabiting the aquatic environment will accumulate various pathogenic microorganisms and chemicals as a result of polluted habitat (Edun *et al*., 2016; Asemota *et al*., 2019). *Bacillus* sp.,

Escherichia coli, *Vibrio* sp. and *Micrococcus* sp. which constitutes part of the indigenous flora of the sea have also been found in sea foods. Food contaminated with high population of these microorganisms could cause cholera, salmonellosis, brucellosis, gastroenteritis, shigellosis, poliomyelitis, amoebiasis, and typhoid fever if it is consumed (Ngozi *et al*., 2020). Periwinkles have a soft skin which predispose it to colonization by various microorganisms. The activities of the microorganisms will cause spoilage when the periwinkles are dead (Ghaly *et al.*, 2010; Nrior *et al.*, 2017). Despite research findings that periwinkle harbour pathogenic microorganisms, some people still prefer to cook periwinkle without removing the shell. This practice might increase the risk of consumers manifesting symptoms of food borne diseases (Nwiyi and Okonkwo, 2013). According to research findings by Nrior *et al*. (2017), the periwinkle or whelk cap, the external body and inner shell fluid of periwinkles are contaminated with high population of many species of microorganisms.

Globally, the consumption of seafood have been identified as one of the sources of foodborne diseases ranging from mild to severe cases. Some common diseases linked to the consumption of contaminated seafood are typhoid fever, cholera, hepatitis and digestive disorder (Adebayo-tayo *et al.*, 2006; Nrior *et al.*, 2017). Generally, pathogenic microorganisms proliferate in seafood because it is rich in nutrients which enhances metabolic activities of the pathogens and increases the risk of foodborne disease transmission (Frazier and Westhoff, 2000). Various pathogenic bacterial and fungal species in high population including viruses have been reported to contaminate periwinkle sold in different localities (Nwiyi and Okonkwo, 2013; Nrior *et al*., 2017; Oluyemi *et al.*, 2019). Majority of the researchers that carried out the study employed standard microbiological methods which have several limitations. The use of molecular characterization methods to identify bacterial and fungal isolates from food samples generate more reliable results than standard microbiological methods (Kelly *et al*., 2020).

Traditionally, periwinkle is harvested by hand picking either from a boat or rock surfaces (Oluyemi *et al*., 2019). It is usually the occupation of women and children. Approximately a day after periwinkles have been shucked, it becomes unsuitable to be consumed unless it is subjected to further processing or appropriate preservation methods (Obire *et al*., 2017). Just like other foods, periwinkle is subjected to food preservation methods such as roasting and drying which reduces the microbial load of the food product and extend the product shelf life. Improvement of organoleptic properties of smoked periwinkle is attributed to chemicals present in wood used in smoking (Akintola *et al.*, 2013). However, the product can be re-contaminated by pathogenic microorganisms in the environment due to poor handling.

Seafood such as periwinkle are highly perishable. In other words, they have a short shelf life. This could be attributed to the chemical effects of atmospheric oxygen and activities of aerobic microorganisms. Drying and smoking are popular preservation methods for periwinkle. A study carried out by Obire *et al*. (2017) analyzed the effectiveness of different preservation methods on the bacterial load of periwinkle and other shelled fish. The order of reduction in bacterial load shows that oven dried > multipurpose dryer dried > smoked dried > sun dried. According to Özpolat *et al*. (2014), vacuum packing is a protection technique employed during refrigeration of seafood. Currently, there are limited studies regarding the use of vacuum packing to extend the shelf life of smoked periwinkle. Therefore, this study is aimed at determining the effect of smoking, polythene and vacuum packing on microbiological quality and shelf life of periwinkle.

2. MATERIALS AND METHODS

2.1 Study Area

Freshly harvested periwinkles (*Tympanotonus fuscatus*) were obtained from Buguma creek located in Asari Toru Local Government Area, Rivers State, Nigeria using sterile polyethene bags. The sample was quickly taken to the Food and Industrial Microbiology Laboratory, University of Port Harcourt for analysis. Figure 1 below shows the map of Buguma Creek.

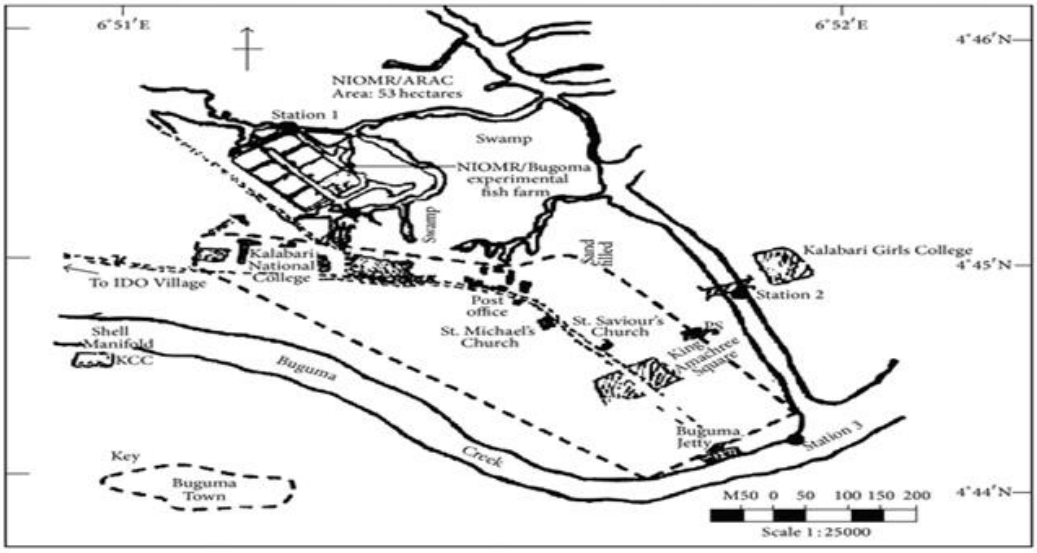

Figure1. *Map showing Buguma Creek*

2.2. Processing of Periwinkle

The periwinkles freshly harvested were processed using the procedure described in Inyang *et al*. (2018) with slight modification. Potable water was used to thoroughly wash the whole body of the periwinkle in order to remove mud and other adhered materials. The periwinkles were put inside aluminum pan and smoked. The periwinkles were smoked using an improved locally manufactured smoking kiln. Mangrove wood was first loaded into the heating chamber and preheated for 15-17 minutes. Afterwards, the periwinkles were poured into a removable sterilized wire mesh tray placed at the centre of the heating chamber for the smoking process. The temperature attained in the heating chamber is between 65 to 70°C which was manually controlled by removing or adding the mangrove wood. The smoking process lasted for 210 minutes. After smoking of the periwinkle is completed, they were allowed to cool to ambient temperature (28±2 ^{o}C). The edible portion (meat) of the periwinkles were manually removed from the shell with the aid of a sterilized stainless pin. The shells were discarded appropriately.

2.3. Packaging and Storage of Periwinkles

Ascetically, hot-smoked periwinkles were divided into three portions labelled 'VP', 'PP' and 'NP'. One portion of the smoked periwinkle labelled 'VP' was vacuum-packaged using a vacuum food sealer Model VS230-IUK and stored at ambient temperature (28 ± 2^{o}C). The vacuum machine expelled air from the transparent packaging material before sealing the smoked periwinkles inside the polythene bag. The second portion of the smoked periwinkle labelled 'PP' was sealed inside a polythene bag without being vacuum-packed. The packaging material used is high density polyethylene (HDPE) bags. The third portion of the smoked periwinkle labelled 'NP' were left without packaging.

2.4. Serial Dilution of Samples

Twenty-five gram (25 g) of blended periwinkle meat samples was transferred into 225 ml 0.1N sterile peptone water to obtain a 10^{-1} homogenate. Ten-fold serial dilution was carried out by transferring 1 ml dilution from the homogenate into the second test tube (dilution 10^{-2}) containing 9 ml sterile peptone water. Stepwise transfer was carried out using sterile pipette for each transfer until dilution 10-6 was achieved.

2.5. Microbiological Analysis

2.5.1. Aerobic Plate Count

Exactly 0.1 ml solution from dilution 10^{-4} and 10^{-5} were plated in duplicate on plate count agar (Biotech, India) supplemented with 1.0 % NaCl using the spread plate method. Isolation of coliforms such as *Escherichia coli* was carried out by inoculating the sample on pre-poured and surface-dried

MacConkey agar (Biotech, India) while thiosulphate-citrate bile- salt-sucrose agar (Biotech, India) was used to isolate *Vibrio* sp. All the inoculated plates were incubated at 37 ºC for 24 h. Enumeration of colony forming units (CFUs) was done by counting representative colonies (30-300). The aerobic plate count for each sample was calculated and expressed as colony forming units per gram (CFU/g) using the formula below.

$$\text{CFU/g} = \text{no. of colonies x } \frac{1}{dilution\ factor} \text{ x } \frac{1}{volume\ plated}$$

2.5.2 Total fungal count

Aseptically, dilutions 10^{-4} and 10^{-5} of each sample was inoculated into freshly prepared Potato Dextrose agar (PDA) medium. The inoculated plates were incubated at room temperature (28±2 ^{o}C) for 5 days and were observed for microbial growth. The total viable count were noted. The total fungal count of each sample was determined using the formula below.

$$\text{CFU/g} = \text{no. of colonies x } \frac{1}{dilution\ factor} \text{ x } \frac{1}{volume\ plated}$$

2.5.3 Purification of the isolates

Representative bacterial and fungal colonies from each culture plate were subcultured by the use of streak method into freshly prepared nutrient agar (NA) and PDA, respectively. The inoculated plates were incubated at 37 °C for 24 h for bacterial growth; room temperature (28±2 ^{o}C) for 5 days for fungal growth. The pure cultures obtained were transferred into agar slants inside Bijoux bottles. The bottles inoculated with pure isolates were stored in a refrigerator until further identification of the isolates were concluded.

2.5.4 Characterization of the bacterial isolates

Motility test and Gram's staining of the bacterial isolates were carried out using the method described by Cheesbrough (2002). Also carried out were biochemical tests which include catalase, citrate, indole, methyl red, Voges-Proskauer, oxidase, hydrogen sulphide production and sugar fermentation. The Bergey's Manual of Determinative Bacteriology was used as a guide for identification of the bacteria (Holt *et al.*, 1994)

2.5.5 Characterization of the fungal isolates

The morphological and microscopic characteristics of fungi isolated from the samples is a guide towards identification of the fungi. Type of mycelium and pigmentation of the sporulating structures were noted. After lactophenol cotton blue staining of the fungal isolates, they were examined microscopically. Identification of the fungal isolates was based on cultural characteristics, morphology of the cells, spores and hyphae.

2.6. Molecular Identification

Molecular characterization of the bacterial isolates were ascertained by sequencing the 16S rRNA of the isolates followed by construction of phylogenetic tree.

2.6.1 DNA Extraction (Boiling method)

The method described by Ugboma *et al*. (2020) was adopted to extract DNA of the isolates. The bacterial isolates were cultured overnight (broth culture) in Luria Bertani (LB). Five milliliter (5 ml) of the broth culture was spun at 14000 rpm in a centrifuge for 3 min. The cells were re-suspended in 500 µl of normal saline and heated at 95 ^{o}C for 20 min. Thereafter, the heated bacterial suspension was cooled on ice. After cooling, the bacterial suspension was spun for 3 min at 14000 rpm. The supernatant containing the DNA was transferred to a 1.5 ml microcentrifuge tube and stored at -20 °C waiting for other downstream reactions.

2.6.2 DNA quantification

The extracted genomic DNA was quantified done using Nanodrop 1000 spectrophotometer using 2 µl of the genetic material.

2.6.3 16S rRNA amplification

The procedure described by Ugboma *et al*. (2020) was implemented in amplifying the 16S rRNA region of the rRNA genes of the isolates using 27F:5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5' CGGTTACCTTG TTACGACTT-3' as primers were used on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 25 microlitres for 35 cycles. The PCR mix used were: the X2 dream taq master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4 M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95 ºC for 5 min; denaturation, 95 ºC for 30s; anealing, 52 ºC for 30 s; extension, 72 ºC for 30 s for 35 cycles and final extension, 72 ºC for 5 min. The product obtained was resolved on a 1% agarose gel at 120V for 20 min and visualized on a blue light transilluminator.

2.6.4 Sequencing

The BigDye Terminator kit was used for the sequencing on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa following the procedure described by Ugboma *et al*. (2020). The sequencing was at a final volume of 10 µL and the components include 0.25 µL BigDye® terminator v1.1/v3.1, 2.25 µL of 5 x BigDye sequencing buffer, 10 µM Primer PCR primer, and 2-10ng PCR template per 100 bp. The sequencing conditions were as follows: 32 cycles of 96 °C for 10 s, 55 °C for 5 s and 60 °C for 4 min.

2.6.5 Phylogenetic analysis

The procedure described by Ugboma *et al*. (2020) was adopted. The bioinformatics algorithm Trace edit was used to edit the sequences obtained. 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 was used to represent the evolutionary history of the taxa analyzed. Jukes and Cantor (1969) method was used to compute the evolutionary distances.

3. RESULTS

Presented in Fig. 1 is the total bacterial count (TBC) and total fungal count (TFC) of freshly harvested periwinkle and non-packaged smoked periwinkle. Depicted in Table 1 and 2 is the characteristics of bacteria isolated from freshly harvested periwinkle (FHP) and non-packaged smoked periwinkles, respectively. The isolates obtained from FHP were *Pseudomonas* sp., *Chryseobacterium* sp., *Enterobacter* sp., *Staphylococcus* sp., *Vibrio* sp., *Providencia* sp., *Bacillus* sp., *Klebsiella* sp. and *Escherichia coli* while *Bacillus* sp and *Providencia* sp. were isolated from the non-packaged smoked periwinkle (NPSP).

Table 3 and 4 shows the characteristics of bacteria isolated from polythene packaged smoked periwinkle (PPSP) and vacuum packaged smoked periwinkles (VPSP), respectively. The isolates obtained from PPSP were *Providencia* sp., *Bacillus* sp., *Staphylococcus* sp., *Chryseobacterium* sp. and *Escherichia coli* while *Providencia* sp., *Bacillus* sp., *Staphylococcus* were isolated from VPSP. Presented in Table 5 is the characteristics of fungi isolated from the periwinkles (*Tympanotonus fuscatus*). The fungal isolates identified were *Nectriaceae* sp., *Aspergillus niger*, *Penicillium* sp.

The effect of smoking, polythene and vacuum packaging on the microbial load of periwinkle stored at ambient temperature (28 ± 2°C) is presented in Table 6. As storage time increased, there was steady increase in total bacterial and fungal count in the stored products.

Plate 1 and 2 depicts the agarose gel electrophoresis of the amplified 16S rRNA of the bacterial isolates and amplified ITS of the fungal isolates from the periwinkles, respectively. The phylogenetic tree showing the evolutionary distance between the bacterial isolates and that which exist between the fungal isolates are presented in Fig. 2 and 3, respectively. Table 7 and 8 shows the accession number assigned to the bacterial and fungal isolates, respectively.

The percentage occurrence of bacterial isolates from freshly harvested periwinkle is presented in Fig. 4. They include *Pseudomonas* sp. (7 %), *Chryseobacterium* sp. (16 %), *Enterobacter* sp. (8 %), *Staphylococcus* sp. (10 %), *Vibrio* sp. (17 %), *Providencia* sp. (9 %), *Bacillus* sp. (18 %), *Klebsiella* sp. (5 %) and *Escherichia coli* (10 %). Fig. 5 shows the percentage occurrence of bacteria isolated

from non-packaged smoked periwinkle. The isolates were *Staphylococcus* sp. (80 %) and *Bacillus* sp. (20 %). The percentage occurrence of bacteria isolated from polythene packaged smoked periwinkle is presented in Fig. 6. They include *Bacillus* sp. (35 %), *Chryseobacterium* sp. (20 %), *Escherichia coli* (14 %), *Klebsiella* sp. (8 %), *Staphylococcus aureus* (12 %) and *Providencia* sp. (11 %). Presented in Fig. 6 is the percentage occurrence of bacteria isolated from vacuum packaged smoked periwinkle which include *Staphylococcus aureus* (60 %), *Proteus* sp. (30 %) and *Bacillus* sp. (10 %).

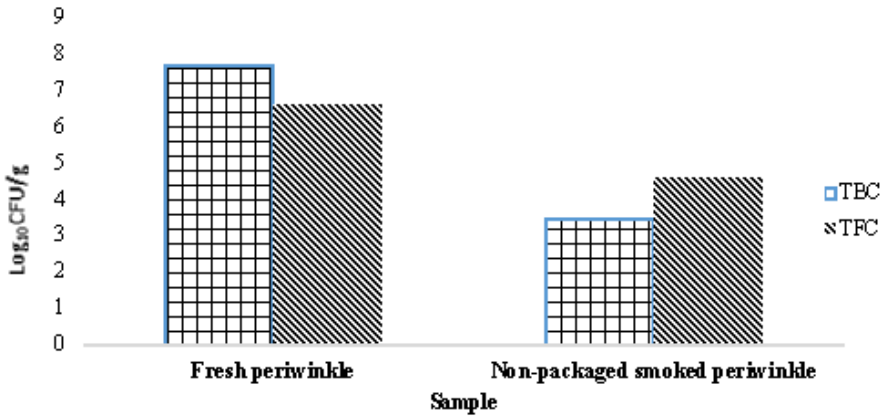

Figure1. *Total bacterial count of freshly harvested periwinkle and non-packaged smoked periwinkle*

Key: TBC – Total bacterial count; TFC – Total fungal count.

Table1. *Characteristics of bacteria isolated from freshly harvested periwinkles (Tympanotonus fuscatus)*

				Biochemical tests								Sugar fermentation tests			Probable isolates
Isolate code	**Cultural characteristics**	**GR**	**Cell morphology**	**Cat.**	**Ox.**	**Ind.**	**MR**	**VP**	**H_2S**	**Mot.**	**Cit.**	**Glu.**	**Lac.**	**Suc.**	
FHP 1	Creamy, circular, mucoid, raised and opaque	-	Rods	+	+	-	-	-	-	+	+	A/G	A	A	*Pseudomonas* sp.
FHP 2	Golden, circular, convex and mucoid.	+	Cocci	+	+	-	-	+		-	+	A	A	-	*Chryseobacterium* sp.
FHP 3	Pale-pink, mucoid, opaque, convex and translucent	-	Rods	+	+	-	+	-	+	+	+	A	-	A	*Enterobacter* sp.
FHP 4	Golden, circular, convex and mucoid.	+	Cocci	+	+	-	-	+	-	-	+	A	A	-	*Staphylococcus* sp.
FHP 5	Yellow, convex, circular and opaque	-	Rods	+	+	+	+	+	-	+	+	A	-	A	*Vibrio* sp.

FHP 6	Irregular, cream, 2 mm, raised colonies	+	Rods	+	-	-	-	-	-	+	+	A/ G	A/ G	A/ G	*Bacillus* spp.
FHP 7	Irregular, cream, 2 mm, raised colonies	-	Rods	+	-	-		+	+	-	+	+	+	+	*Providencia* sp.
FHP 8	Irregular, cream, 2 mm, raised colonies	+	Rods	-	-	-	-	-	-	-	-	A/ G	-	A/ G	*Klebsiella* sp.
FHP 9	Pink, mucoid, convex and circular.	-	Rods	+	-	+	+	-	-	+	-	+	+	-	*Escherichia coli*

Key: FHP =Freshly harvested periwinkle, GR-Gram reaction; + = Positive, - = Negative; Cat-Catalase test; Ox-Oxidase test; Ind-Indole test; MR- Methyl red test; VP-Voges-Proskauer; Cit-Citrate utilization; Mot-Motility test; H_2S-Hydrogen sulphide production; Glu-Glucose; Lac-Lactose; Suc-Sucrose.

Table2. *Characteristics of bacteria isolated from non-packaged smoked periwinkles (Tympanotonus fuscatus)*

				Biochemical tests								**Sugar fermentation tests**			**Probable isolates**
Isolate code	Cultural characteristics	GR	Cell morphology	Cat.	Ox.	Ind.	MR	VP	H_2S	Mot.	Cit.	Glu.	Lac.	Suc.	
NPSP1	Irregular, cream, 2 mm, raised colonies	+	Rods	+	-	-	-	-	-	+	+	A/ G	A/ G	A/ G	*Bacillus* sp.
NPSP2	Irregular, cream, 2 mm, raised colonies	-	Rods	+	-	-	-	+	+	-	+	+	+	+	*Providencia* sp.

Key: NPSP-Non-packaged smoked periwinkle; GR-Gram reaction; + = Positive; - = Negative; Cat-Catalase test; Ox- Oxidase test; Ind-Indole test; MR-Methyl red test; VP- Voges-Proskauer test; Cit-Citrate utilization; Mot-Motility test; H_2S-Hydrogen sulphide production; Glu-Glucose; Lac- Lactose; Suc-Sucrose.

Table3. *Characteristics of bacteria isolated from polythene packaged smoked periwinkle (Tympanotonus fuscatus)*

Isolate Code	**Cultural characteristics**	**Morphology Characteristics**		**Biochemical tests**								**Sugar fermentation test**			**Probable isolates**
		GR	**CM**	**Cat.**	**Ox.**	**Ind.**	**MR**	**VP**	**H_2S**	**Mot.**	**Cit.**	**Glu.**	**Lac.**	**Suc.**	
PSP 1	Irregular, cream, 2 mm, raised colonies	+	Rods	+	-	-	-	-	-	+	+	A/ G	A/ G	A/ G	*Bacillus* sp.
PSP 2	Golden, circular,	-	Cocci	+	+	-	-	+		-	+	A	A	-	*Chryseobac*

	convex and mucoid														*teruim* sp.
PSP 3	Pale-pink, mucoid, opaque, convex and translucent	+	Rods	+	+	-	+	-	+	+	+	A	-	A	*Escherichia coli*
PSP 4	Golden, circular, convex and mucoid	+	Cocci	+	+	-	-	+		-	+	A	A	-	*Staphylococcus* sp.
PSP 5	Pale-pink, mucoid, opaque, convex and translucent	-	Rods	+	+	-	+	-	+	+	+	A	-	A	*Providencia* sp.
PSP 6	Irregular, cream, 2 mm, raised colonies	+	Rods	+	-	-	-	-	-	+	+	A/G	A/G	A/G	*Bacillus* sp.

Key: PSP= Polythene packaged smoked periwinkle, GR= Gram reaction, CM= Cell morphology, + = Positive, - = Negative, Cat = Catalase test, Ox = Oxidase test, Ind = Indole test, MR=Methyl red test, VP = Voges-Proskauer test, Cit = Citrate utilization, Mot = Motility test, H_2S = Hydrogen sulphide production, Glu = Glucose, Lac = Lactose, Suc = Sucrose.

Table4. *Characteristics of bacteria isolated from vacuum packaged smoked periwinkles (Tympanotonus fuscatus)*

Isolate Code	**Cultural characteristics**	**Morphology characteristics**		**Biochemical tests**								**Sugar fermentation test**			**Probable isolates**
		GR	**CM**	**Cat.**	**Ox.**	**Ind.**	**MR**	**VP**	**H_2S**	**Mot.**	**Cit.**	**Glu.**	**Lac.**	**Suc.**	
VPSP1	Pale-pink, mucoid, opaque, convex and translucent	-	Rods	+	+	-	+	-	+	+	+	A	-	A	*Providencia* sp.
VPSP2	Creamy, circular, mucoid, raised and opaque	-	Rods	+	+	-	-	-	-	+	+	A/G	A	A	*Staphyloccus* sp.
VPSP3	Irregular, cream, 2 mm, raised colonies	+	Rods	-	-	-	-	-	-	-	-	A/G	-	A/G	*Bacillus* sp.

Key: VPSP -Vacuum packaged smoked periwinkle; GR- Gram reaction; CM-Cell morphology; + = Positive; - = Negative; Cat- Catalase test; Ox-Oxidase test; Ind-Indole test;

MR-Methyl red test; VP-Voges-Proskauer test; Cit-Citrate utilization; Mot-Motility test; H_2S-Hydrogen sulphide production; Glu-Glucose; Lac-Lactose; Suc-Sucrose.

Table5. *Characterization and identification of fungi isolated from the periwinkles (Tympanotonus fuscatus)*

Sample	Isolate code	Cultural characteristics	Cell morphology/Microscopy	Tentative genera
Freshly harvested periwinkle (control)	FHP1	Black surface with white border and cream reverse	Septate hyphae with long smooth conidiophores and rough dark conidia	*Aspergillus niger*
	FHP2	White colonies with grey center	Smooth walled conidiophores	*Penicillium* sp.
	FHP3	Whitish and cottony colony	Branched conidiophores with smooth conidia in chains or pairs	*Nectriaceae* sp.
Smoked periwinkle	NPSP1	Black surface with white border and cream reverse	Septate hyphae with long smooth conidiophores and rough dark conidia	*Aspergillus niger*
Polythene packaged smoked periwinkle	PPSP1	Black surface with white border and cream reverse	Septate hyphae with long smooth conidiophores and rough dark conidia	*Aspergillus niger*
Vacuum packaged smoked periwinkle	VPSP2	Whitish and cottony colony	Branched conidiophores with smooth conidia in chains or pairs	*Nectriaceae* sp.

Key: FHP-Freshly harvested periwinkle; NPSP-Non-packaged smoked periwinkle; PPSP-Polythene packaged smoked periwinkle; VPSP-Vacuum packaged smoked periwinkle.

Table6. *Effect of smoking, polythene and vacuum packaging on the microbial load of periwinkle stored at ambient temperature (28±2°C)*

Sample	Storage time (Wks)	TBC (log_{10}CFU/g)	TFC (log_{10}CFU/g)
Non-packaged smoked periwinkle	0	3.45	3.58
	2	8.68	7.67
Polythene packaged smoked periwinkles at ambient temperature (28 ± 2°C)	0	3.45	3.58
	2	5.56	4.67
	4	6.64	5.70
	6	8.75	6.88
Vacuum packaged smoked periwinkles at ambient temperature (28 ± 2°C)	0	3.45	3.58
	2	4.53	3.54
	4	3.58	3.56
	10	3.63	3.58
	12	4.67	3.59
	16	5.71	4.63

Key: TBC-Total bacterial count; TFC-Total fungal count

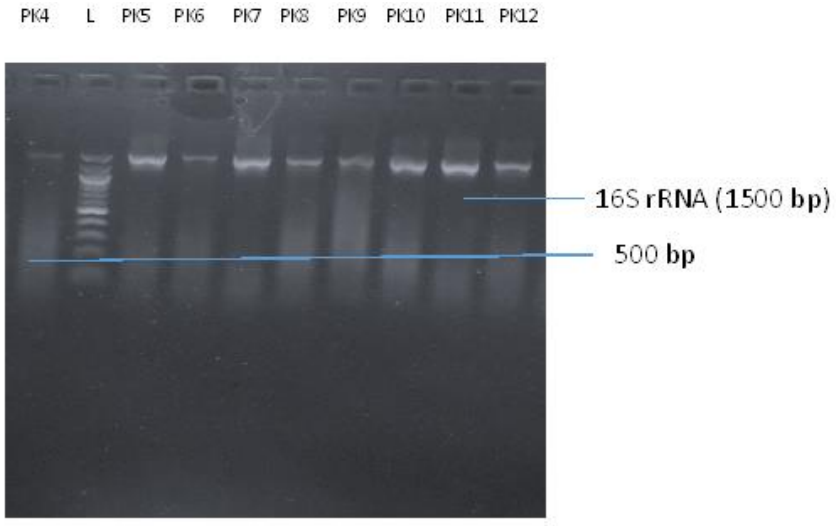

Plate1. *Agarose gel electrophoresis of the amplified 16S rRNA. Lanes PK4-PK12 represent the amplified 16S rRNA gene at 1500 bp while lane L represents the 100 bp molecular ladder.*

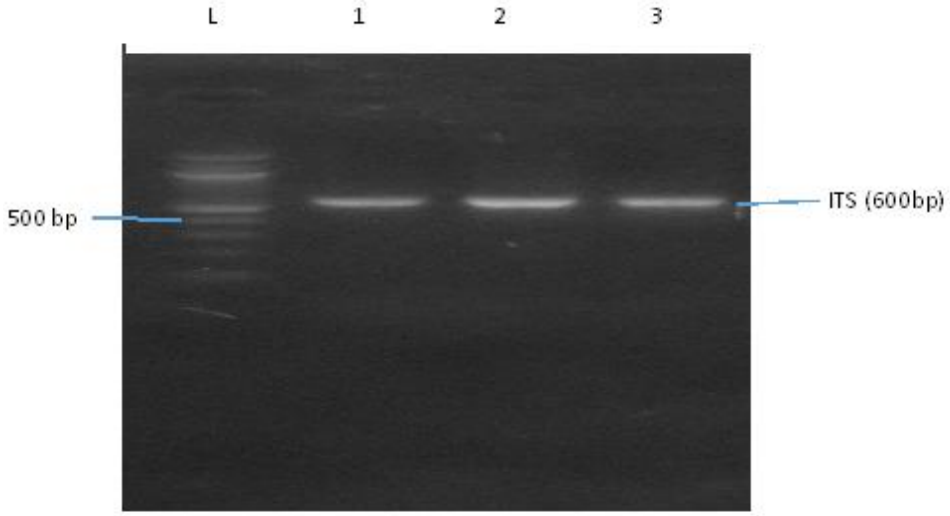

Plate2. Agarose gel electrophoresis showing the amplified ITS of the fungal isolates. Lane 1-3 represent the ITS bands at 600bp while lane L represents the 100bp molecular ladder.

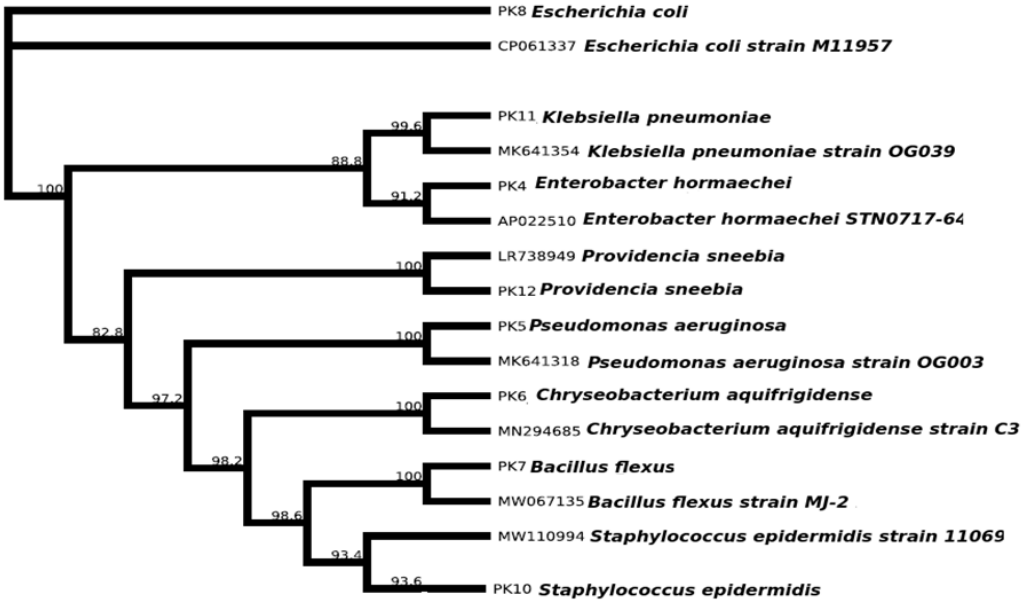

Figure2. *Phylogenetic tree showing the evolutionary distance between the bacterial isolates*

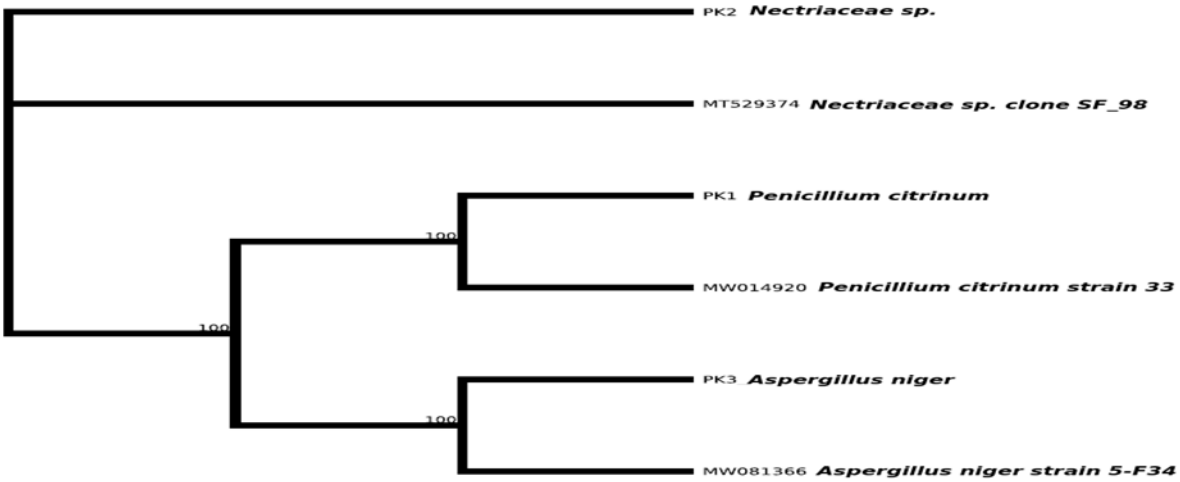

Figure3. *Phylogenetic tree showing the evolutionary distance between the fungal isolates*

Table7. *Accession number of the bacterial isolates*

Isolate Code	Accession number	Similarity index (%)	Bacterial species
PK4	MW578427	91.2	*Enterobacter hormaechel*
PK5	MW578428	100	*Pseudomonas aeruginosa*
PK6	MW578429	100	*Chryseobacterium aquifrigidense*
PK7	MW578430	100	*Bacillus flexus*
PK8	MW578432	100	*Escherichia coli*
PK10	MW578431	93.6	*Staphylococcus epidermidis*
PK11	MW578433	99.6	*Klebsiella pneumoniae*
PK12	MW578434	100	*Providencia sneebia*

Table8. *Accession number of the fungal isolates*

Isolate Code	Accession number	Similarity index (%)	Fungal species
PK1	MW578418	100	*Penicillium citrinum*
PK2	MW578419	100	*Nectriaceae* sp.
PK3	MW578426	100	*Aspergillus niger*

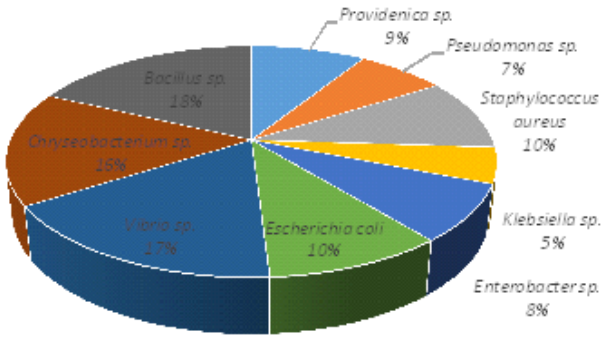

Figure4. *Percentage occurrence of bacteria isolated from freshly harvested periwinkle*

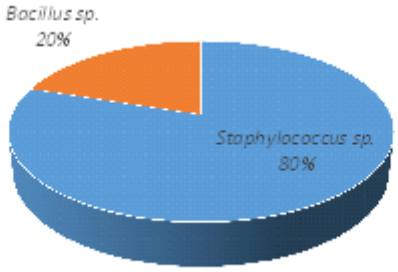

Figure5. *Percentage occurrence of bacteria isolated from non-packaged smoked periwinkle*

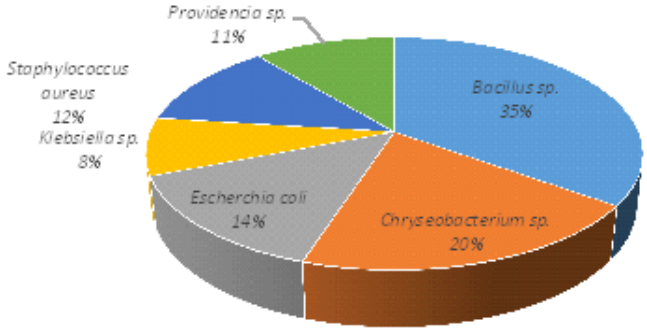

Figure6. *Percentage occurrence of bacteria isolated from polythene packaged smoked periwinkle*

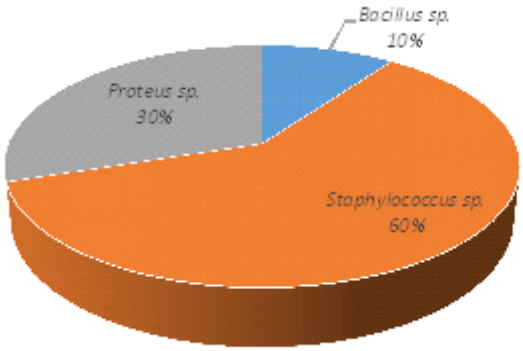

Figure7. *Percentage occurrence of bacteria isolated from vacuum packaged smoked periwinkle*

4. DISCUSSION

The result obtained from this study shows that total bacterial count (TBC) and total fungal count (TFC) of freshly harvested periwinkle (*Tympanotomus fuscatus*) is 7.68 and 6.6 $\log_{10}$CFU/g while the corresponding values for non-packaged smoked periwinkle is 3.45 and 4.58 $\log_{10}$CFU/g, respectively. The reduction in bacterial and fungal count in the non-packaged smoked periwinkle compared with the freshly harvested periwinkle could be attributed to antimicrobial effect of the smoking process. This result is in agreement with a related study carried out by Kumolu-Johnson *et al*. (2010) which reported a reduction in total coliform count in fish (*Clarias gariepinus*) after undergoing smoking process. In a related study, Chika and Mercy (2019) reported that boiled periwinkle had lower bacterial population than what was encountered in freshly harvested periwinkle. According to Abraha *et al.* (2018), smoking fish generate heat and antimicrobial smoke chemicals such as phenols and formaldehydes which attack microorganisms. Thus, reduction in water activity as a result of smoking fish slow down spoilage and extend shelf life of the sample.

A total of nine (9) bacterial genera which include *Bacillus*, *Staphylococcus*, *Klebsiella*, *Escherichia*, *Providencia*, *Pseudomonas*, *Vibrio*, *Enterobacter* and *Chryseobacterium* were isolated from freshly harvested periwinkles. Consumption of periwinkles contaminated with these pathogenic bacterial species without subjecting it to a kill step such as smoking in order to reduce most of the microorganisms present in the periwinkles could have serious health implications. Most of the bacterial genera isolated from the periwinkles were also reported by Adesanya *et al.* (2021) and Adebayo-tayo *et al.* (2006) in separate studies that involved bacteriological quality assessment of periwinkle. According to Obire *et al*. (2017), the bacteria flora of fresh molluscan shellfish which include periwinkle is largely dependent on the environment where they were harvested as well as handlers of the product and not the periwinkle. The presence of enteric microorganisms in freshly harvested periwinkle is a strong indication that aquatic environment where they were harvested is polluted with untreated sewage and faecal waste (Adebayo-tayo *et al.*, 2006).

Findings from this study show that three (3) fungal species were isolated from both freshly harvested and smoked periwinkle. The fungal isolates were *Aspergillus* sp., *Nectriaceae* sp. and *Penicillium* sp. The possible sources of *Aspergillus* and *Penicillium* sp. in the periwinkle are water, soil and air where the spores of the fungi are commonly found. According to Lombard *et al.* (2015), various human and plant pathogens of significance make up the ascomycete family *Nectiaceae* (Hypocreales). Various species belonging to the family *Nectiaceae* have useful commercial applications in some industries as biodegraders and biocontrol agents. In a related study, Ngozi *et al.* (2020) reported that *Aspergillus niger*, *A. flavus*, *Penicillium* sp. and *Mucor* sp. were present in dried periwinkle sold in a local market.

Escherichia coli is part of the intestinal flora of humans and vertebrates. In humans, some species of *Escherichia* are associated with infantile diarrhea and newborn meningitis. It has been reported that some species of *Enterobacter* are responsible for septicemia and neonatal meningitis (Adebayo-tayo *et al*., 2006). The production of enterotoxins is associated with some strains of *Staphylococci* and *Bacillus* which poses a serious threat to consumers of food containing large population of these organisms (Ngozi *et al*., 2020). According to Obire *et al.* (2017) toxin production by *Staphylococcus*

aureus which is a mesophilic organism occurs when the population of the bacterium exceed 10^6 CFU/g in an appropriate temperature. *Pseudomonas aeruginosa* is a common opportunistic pathogen ubiquitous in nature. It is present in some blood infections, burns, and wounds. Since the aquatic environment where periwinkle is harvested determines its bacteria flora, the presence of *Pseudomonas* sp. in periwinkle could be traced to individuals bathing inside the water with open wounds or other infections (Omenwa *et al*., 2011). *Vibrio* sp. is associated with symptoms of gastroenteritis. The symptoms range from mild diarrhea to profuse watery diarrhea which is the classical cholera. Likely sources of *Vibrio* sp. in periwinkle are domestic and industrial waste dumped inside the water body where the periwinkles were harvested, bathers and individuals using the water body for other recreational activities (Nrior *et al*., 2017). Studies have shown that *Chryseobacterium* species are found in diverse habitats. This microorganism has been isolated from soils, freshwater creek and lakes. It is associated with diverse animals such as mosquitoes and fish. Some species of *Chryseobacterium* sp. are capable of causing human infections (Loch and Faisal, 2015). According to Maduka *et al*. (2021), *Klebsiella* sp. is associated with diarrhea, pneumonia, septicaemia, pyogenic infections and urinary tract infections.

Providencia and *Bacillus* were the only bacterial genera isolated from non-packaged smoked periwinkle stored for two (2) weeks at ambient temperature (28±2 ^oC). A total of five (5) bacterial genera namely *Chryseobacteruim* sp., *Staphylococcus* sp., *Escherichia coli Providencia* sp and *Bacillus* sp were isolated from polythene packaged smoked periwinkle (PPSP) stored at ambient temperature (28±2 ^oC) for six (6) weeks. Worthy to note is that a total of three (3) bacteria species namely *Providencia* sp., *Staphylococcus* sp. and *Bacillus* sp. were isolated from vacuum packaged smoked periwinkle (VPSP) stored for sixteen (16) weeks. This result is an indication that vacuum packaging created the most unfavourable condition for bacterial species to survive in the smoked periwinkle notwithstanding longer period of storage compared with polythene packaging and non-packaging of smoked periwinkle stored for a shorter period. Research findings by Ochieng *et al*. (2015) stated that total viable count (TVC) of chilled vacuum packing of fish had lower total viable count than fish packed in gunny bag, polythene air-packed fish, polythene air-packaged and ambient stored Gunny bag.

Further characterization of the bacterial isolates using molecular methods revealed that *Enterobacter* sp. share 91.2 % similarity with *Enterobacter hormaechei* STN0717-64, *Staphylococcus* sp. share 93.6 % similarity with *Staphylococcus epidermidis* strain 11069, *Klebsiella* sp share 99.6 % similarity with *Klebsiella pneumoniae* strain OG039, *Escherichia coli* share 100 % similarity with *Escherichia coli* strain M11957, *Providencia* sp. share 100 % similarity with *Providencia sneebia*, *Pseudomonas aeruginosa* share 100 % similarity with *Pseudomonas aeruginosa* strain OG003, *Chryseobacterium aquifrigidense* share 100 % similarity with *Chryseobacterium aquifrigidense* strain C3 while *Bacillus flexus* share 100 % similarity with *Bacillus flexus* Mj-2.

Molecular characterization of *Aspergillus* sp., *Penicillium* sp. and *Nectriaceae* sp isolated from the periwinkles revealed they have 100 % similarity with *Aspergillus niger* strain 5-F34 *Penicillium citrinum* strain 33 and *Nectriaceae* sp. clone SF_98, respectively. In a related study, Kelly *et al*. (2020) identified fungi isolated from periwinkle (*Tympanotonus fuscatus*) using standard microbiological methods. Further characterization of the fungal isolates using molecular methods revealed that *Candida* sp. showed 99.65 % similarity with *Meyerozyma guilliermondii*; *Fusarium* sp showed 99.38 % similarity with *Fusarium oxysporum* isolate E-2251 while *Aspergillus* sp. showed 96.23 % similarity with *Aspergillus terreus* isolate A2S4_D50. This result is not in agreement with the findings from this study. Environmental factors such as pollution and seasonal variations could be attributed to the differences in fungal species isolated from the periwinkles.

During storage of non-packaged smoked periwinkle (NPSP), polythene packaged smoked periwinkles (PPSP) and vacuum packaged smoked periwinkles (VPSP) at ambient temperature (28 ± 2 ^oC), there was increase in total bacterial count (TBC) and total fungal count (TFC). Among all the samples stored at ambient temperature, the NPSP had a striking result in the sense that after two (2) weeks of storage at ambient temperature, the TBC and TFC of the sample at least doubled. At week 0 and 2, the TBC of non-packaged smoked periwinkle was 3.45 and 8.68 $\log_{10}$CFU/g while the corresponding values for TFC was 3.58 and 7.67 $\log_{10}$CFU/g, respectively. Exposure of non-packaged smoked periwinkle to air harbouring millions of microorganisms for two (2) weeks could be the reason behind

the tremendous increase in population of TBC and TFC of the sample compared with the values recorded for VPSP and PPSP. The total fungal count of VPSP and PPSP during storage were within the range of 3.58-4.63 and 3.58-6.88 $\log_{10}$CFU/g while the corresponding values for TBC were 3.45-5.71 and 3.45-8.75 $\log_{10}$CFU/g, respectively. This result is an indication that vacuum packing is more effective than polythene packing in inhibiting growth of microorganisms during storage of smoked periwinkle. To ensure the safety of consumers of shell fish, The International Commission on Microbiological Specifications for Food (ICMSF) recommend that total plate count (TPC) of shellfish should not exceed 5 $\log_{10}$CFU/g (Adebayo-tayo *et al.*, 2006). According to Amadi *et al*. (2014), a standard threshold of 10^4 CFU with respect to total fungal count of food should not be exceeded for it to be considered safe for human consumption. Considering the limits set by both standards, vacuum-packed smoked periwinkle stored for 12 Weeks; polythene packaged and non-packaged smoked periwinkle stored for some days (< 2 weeks) are safe for human consumption.

Among the bacterial isolates obtained from freshly harvested periwinkle, *Bacillus* sp. (18 %) and *Klebsiella* sp. (5 %) had the highest and least frequency of occurrence, respectively. The highest and least frequency of occurrence of bacteria isolated from polythene packaged smoked periwinkle involved *Bacillus* sp. (35 %) and *Klebsiella* sp. (8 %), respectively. However, *Staphylococcus* sp. (80 %) had the highest frequency of occurrence in non-packaged smoked periwinkle while *Bacillus* sp. (20 %) had the least. Among the bacterial isolates obtained from vacuum packaged smoked periwinkle, *Staphylococcus* sp. (60 %) and *Bacillus* sp. (10 %) had the highest and least frequency of occurrence, respectively. The dominance of *Bacillus* sp. in polythene packaged smoked periwinkle and freshly harvested periwinkle could be attributed to prevalence of the spore-forming bacterium in the environment. As for the predominance of *Staphylococcus* sp. in non-packaged smoked periwinkle and vacuum packaged smoked periwinkle, it could be attributed to improper handling and cross contamination of the product.

5. CONCLUSION

Total bacterial and fungal count of freshly harvested periwinkle were higher than the values reported for non-packaged smoked periwinkles. A total of nine bacterial species were isolated from freshly harvested periwinkle while a lesser number were encountered in polythene packaged, vacuum packaged and non-packaged smoked periwinkle during the storage period at ambient temperature. Also isolated from the periwinkles were three fungal species. The bacterial isolates identified were *Vibrio* sp., *Staphylococcus epidermidis* strain 11069, *Escherichia coli* strain M11957, *Klebsiella pneumoniae* strain OG039, *Providencia sneebia*, *Pseudomonas aeruginosa* strain OG003, *Chryseobacterium aquifrigidense* strain C3, and *Bacillus flexus* Mj-2 while the fungal isolates include *Aspergillus niger* strain 5-F34 *Penicillium citrinum* strain 33 and *Nectriaceae* sp. clone SF_98. With reference to maximum total plate count of 5 $\log_{10}$CFU/g for shellfish recommended by International Commission on Microbiological Specifications for Food (ICMSF) and standard threshold of 10^4 CFU for total fungal count of food, the shelf life of non-packaged and polythene packaged smoked periwinkle is less than two weeks while that of vacuum packaged smoked periwinkle is fourteen weeks. The bacteria genera isolated from periwinkles (NPSP and VPSP) which had the highest and least frequency of occurrence were *Staphylococcus* sp. and *Bacillus* sp., respectively. Similarly, the bacteria genera isolated from periwinkles (FHP and PPSP) which had the highest and least frequency were *Bacillus* sp. and *Klebsiella* sp., respectively.

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