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Occurrence of virulent and antibiotic resistant *Staphylococcus* species in ready-to-eat *Rhynchophorus phoenicis* and *Archachatina marginata* vended along the Port Harcourt-Bayelsa route

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This study was aimed at determining the occurrence of *Staphylococcus* species in ready-to-eat (RTE) *Rhynchophorus phoenicis* (edible larvae) and *Archachatina marginata* (land snail) vended along Port Harcourt-Bayelsa route. Eighty samples from four locations were analysed employing standard techniques for proximate and microbiological analyses; virulence determination and antibiotic susceptibility. Data were statistically analyzed using ANOVA and t-test. The mean proximate results revealed the presence of protein (26.01/13.6%), lipid (18.9/3.88%), fibre (5.12/2.01%), ash (3.40/1.11%), moisture (13.47/59.5%) and carbohydrate (32.43/20.07%) for edible larvae/snail, respectively. Of the 80 samples examined, 33 (41.25%) and 52 (65.00%) had total viable bacterial counts and total staphylococcal counts above acceptable microbiological limits, respectively for RTE foods. Seven of the 81 characterized *Staphylococcus* produced the expected band of 950 bp with *sea* virulent genes while three produced expected bands of 550 bp with *seb* virulent genes. Three *Staphylococcus aureus* strains from edible larvae harboured both virulent genes. The virulent genes bearing *Staphylococcus* were 100% resistant to augmentin, ceftazidime and cloxacillin but showed varying resistance against ceftriaxone (57.14%), cefuroxime (28.57%), vancomycin (42.86%), oxacillin (42.86%) and cefoxitin (42.86%). The study showed that these RTE foods are potential sources of staphylococcal food poisoning in commuters; hence, food vendors need to conform to standard practice.

Key words: Edible larvae, land snail, staphylococcal enterotoxin A (*sea*), virulence.

INTRODUCTION

Ready-to-eat foods (RTE) may be raw, minimally cooked or cooked foods and are vended for instant consumption without additional preparation. The usage of RTE foods prepared and sold by the street vendors has expanded in several African countries, including Nigeria (Makelele et al., 2015). There are varieties in the form of snacks,

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meats, salads, fruits and also beverages. Ready-to-eat foods have gained a lot of popularity such that they are available in the markets, roadsides and restaurants. Food vendor services is still on the increase, emerging to a new form referred to as migratory food vending especially on highways where RTE foods are highly exposed to microbial hazards. The pervasive routine of open presentation and exposure of ready-to-eat foods and the deficiencies of the existing food safety frameworks pose danger to the general wellbeing of commuters who patronize these foods. In Nigeria, African palm tree weevil larva (*Rhyncophorus phoenicis*) and African land snail meat (mainly *Archachatina marginata*) are regular edible insects and mollusks, respectively that are vended by migratory food vendors on the busy roads of Niger Delta states like Bayelsa, Delta, Edo, Imo, Rivers and also upper Cross-River Basins (Arene et al.,1999; Ekrakene and Igeleke, 2007). African palm tree weevil larva is popularly called edible larvae and it is widely recognized and prevalent in Tropical Africa (Tambe et al., 2013). It is principally sufficient in fat (66.61%) and protein (21.06%) (Womeni et al., 2012). It is a cherished delicacy not only in Southern Nigeria but other African countries like Cameroon. In ethnic/local languages, it is called 'eruru' in Ibo land, 'orhu' in Edo, 'odon' in Delta and 'awon' or 'ekuku' in Yoruba land. African palm weevil can be consumed raw, boiled, fried or roasted. Unlike other kinds of edible insects, African palm weevils are available year-round in variable quantities and also commonly seen in the southern parts of Nigeria rainforest zone while in Cameroon it is ubiquitous in moist swampy forest and Savannah zones (Fogoh et al., 2015). African land snails are domicile extensively in African countries like Nigeria, Ghana and also Central and Southern Africa where the climatic conditions are appropriate for rapid reproduction (Herbert and Kilburn, 2004). The two different land snails; *A. marginata* and *Achatina achatina* are well known edible snail groups in bounty in these localities (Ajayi et al., 2009). A report from USDA (2006) describes snail meat as top-notch sustenance, which is adequate in protein, lesser lipids and a decent wellspring of iron. Hence, these foods are patronized by travellers regardless of how they were processed by the migratory food vendors because of their nutritive properties. However, these are vehicles of transferring pathogens of general health significance to consumers since they may be inadequately processed or poorly handled during packaging and at point of sales. Although no reports of food borne illnesses have been linked with raw and roasted *R. phoenicis* larvae; they are nevertheless associated with a number of microorganisms, namely: *Staphylococcus aureus, Bacillus* species, *Proteus* species, *Lactobacillus plantarum, Pseudomonas* species, *Escherichia coli, Enterococcus* species, *Proteus vulgaris, Serratia* species, *Acinetobacter* species, *Enterobacter* species, *Micrococcus* species, *Salmonella* species, *Listeria* species, *Aspergillus* species, *Penicillium* species, *Cladosporium* species, *Fusarium* species, *Rhizopus* species and *Mucor* species (Wachukwu et al., 2002; Ekrakene and Igeleke, 2007; Oranusi and Braide, 2012; Amadi et al., 2014; Ebenebe and Okpoko, 2015; Daniel and Onilude, 2017).

Land snails have close association with microorganisms living in the soil environment whether wild or cultured, this affinity with snail and microorganisms is due to habitat filth, sewage and rotten materials (Agbonlahor et al., 1994). The microbial biota of snail in their unprocessed state is a contribution of their environment microbial community. Snails living in regular habitat can harbour pathogenic Enterobacteriaceae; at least 40 genera are listed including *Salmonella, Proteus, Serratia, Enterobacter*, *Citrobacter, Pseudomonas* and *Klebsiella* spp. (Fagbuaro et al., 2006). A number of authors have reported the occurrence of *E. coli, Klebsiella* spp., *Salmonella* spp., *Enterobacter* spp., *Shigella* spp., *Citrobacter* spp., *Pseudomonas* spp., *Staphylococcus* spp., *Micrococcus* spp., *Proteus* spp., *Bacillus subtilis, Bacillus cereus, Lactobacillus* spp. and *Listeria monocytogenes* in African land snail species (Adegoke et al., 2010; Nwuzo et al., 2016; Nyoagbe et al., 2016).

Staphylococcal food poisoning (SFP), caused by enterotoxin-producing *S. aureus* strains is an important foodborne illness in some countries (Wieneke et al., 1993; Le Loir et al., 2003). *S. aureus* are linked to many clinical cases (Adegoke and Komolafe, 2009). Thomas et al. (2007) described 19 types of staphylococcal enterotoxins (SEs); Group 1, classical emetic toxins: staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB), staphylococcal enterotoxin C1 (SEC1), staphylococcal enterotoxin C boy (SECbov), staphylococcal enterotoxin D (SED) and staphylococcal enterotoxin E (SEE), responsible for 95% food poisoning in man.

It is common to hear persons who changed locations complaining of stomach upset not usually linked to ready-to-eat foods consumed along the route because of varying incubation periods of food borne pathogens. The study was aimed at determining the occurrence of *Staphylococcus* spp. in ready-to-eat edible larvae (*R. phoenicis*) and land snail meats (*A. marginata*) vended along Port Harcourt-Bayelsa route.

MATERIALS AND METHODS

Sample collection

A total of eighty ready-to-eat food samples comprising forty roasted edible larvae and forty land snail meats were purchased at different locations [Edible larvae (Mbiama, Tombia, Ekeki and Opokuma) and snail meats (Mbiama, Choba-Emohua, Ahoada and Opokuma)]. These ready-to eat foods were partially exposed and poorly packaged (Figures 1 and 2). The samples were aseptically stored in well labelled and sealed sterile transparent bags and transported

Figure 1. Ready-to-eat land snail meats (*Archachatina marginata*).

Figure 2. Roasted ready-to-eat edible larvae (*Rhynchophorus phoenicis*).

immediately to the Microbiology laboratory for analyses.

Proximate analyses of the RTE food samples

The proximate parameters: ash content, moisture content, crude protein, crude lipid, crude fibre and total carbohydrate of the ready-to-eat samples were determined as described by Association of Official Analytical Chemists (AOAC, 2000). Crude protein was analysed using Kjedahl method, adopted from the procedures of James (1995) and Chang (2003). The percentage of the total nitrogen derived was converted to crude protein by multiplying by 6.25 (that is N × 6.25). Crude fat was performed by extraction with petroleum ether solvent. The defatted samples were analysed for ash content. Carbohydrate content of the food was determined by percentage difference in sum of other parameters (ash content, crude protein, crude fibre, and crude fat and moisture contents) from 100. Each parameter was conducted in duplicates.

Microbiological analyses

Sample preparation

Twenty-five grams of the food sample was transferred to the sterile blender (Philip, HR2001, China) macerated/diluted with 225 ml sterile peptone water (Oxoid, Basingstoke, UK) for 2 min. Serial dilution was done on the homogenized stock solution up to 10^{-6} as described by Makelele et al. (2015).

Determination of total viable counts

Aliquot (0.1 mL) of each of the selected sample dilutions was

Table 1. Primers of target genes for isolated *S. aureus* strains sequence by Kamarehei et al. (2013).

Gene	Primer sequence 5’ → 3’	Amplicon size (bp)
sea	F5´-TTGGAAACGGTTAAAACGA-3' R 5´-GAACCTTCCCATCAAAAAC-3'	120
seb	F5´-TCGCATCAAACTGACAAAC-3´ R5´-GCAGGTACTCTATAAGTGC-3´	409

aseptically spread plated on the sterile solidified nutrient agar (NA: TM, Rajasthan, India) in triplicate. The inoculum was allowed to be absorbed into the agar plate, inverted and incubated at 30°C for 24 h. After incubation, average number of colony counts of the same dilution was recorded and colony forming units per gram (CFU/g) obtained and expressed as logarithm (log_{10} CFU/g) (Harrigan and Mc-Cance, 1990; Cheesbrough, 2010).

Enumeration and isolation of S. aureus

Aliquot (0.1 mL) of the selected sample dilutions were inoculated on freshly prepared, dry surface mannitol salt agar (MSA; Oxoid, Basingstoke, UK) and spread carefully on the entire surface of the agar plates. The inocula were allowed to absorb, thereafter, incubated at 30±2°C for 48 h. A colour change in the agar from red to yellow (fermentation of mannitol) indicated presumptive *S. aureus* colonies. Hence, mannitol positive colonies surrounded by yellow halo were counted (Makelele et al., 2015). *Staphylococcus* discrete colonies were counted, recorded and used to obtain staphylococci colony forming unit per gram and expressed in logarithm log_{10} CFU/g (Lancette and Benett, 2001).

Molecular characterization of isolated *S. aureus*

DNA extraction

The extraction of DNA was by boiling method described by Eruteya et al. (2014) and Vendruscolo et al. (2017). For DNA extraction, aliquot of 0.5 mL of 5 mL overnight Luria Bertani (LB) broth culture of bacterial isolates was transferred to 2 mL Eppendorf tubes. The cells were centrifuged (Dupont Sorvall MC-12V, biomedical division, USA) at 16099 g. for 3 min after addition of sterile distilled water. The supernatant was discarded while the harvested cells in the residue were vortexed (Vortexer VWR G-560, Scientific Industries, USA) and re-centrifuged after re-suspension in 500 µL of normal saline. DNA elution buffer (500 µL) was then added to the residues after discarding the supernatants and spun again. Thereafter, 300 µL DNA elution buffer was added to the residues and vortexed for about 3 min, and then heated with a heating block (Wealtec HB-2, Wealtec Corp, USA) at 95°C for 20 min. The heated bacterial suspensions were fast cooled on ice to -20°C for 10 min and spun for 3 min at 16099 g. The solutions containing the extracted genomic DNA (200 µL) were moved to 1.5 mL micro-centrifuge tubes and kept at -20°C in the deep freezer for further downstream reactions.

The extracted DNA of the isolates were quantified using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Inqaba Biotechnological Industries Ltd Pretoria, SA) which was used to measure the DNA concentration in ng/µL.

Amplification of sea genes for S. aureus strains

The *sea* genes from the suspected enterotoxigenic *S. aureus* isolates were amplified using the *sea* primers (Table 1) on DNA thermal cycler (Gene^{Amp} PCR system) ABI 9700 Applied Biosystems, SA) at final volume of 30 µl for 35 cycles. The PCR mix included: 15 µL X2 Dream Taq Master Mix (Inqaba Biotechnological Industries Ltd, Pretoria, South Africa), the forward and reverse primers at a concentration of 0.4 µM each and 50 ng of the extracted DNA template. The PCR conditions were as follows: Initial denaturation at 95°C for 5min; denaturation at 95°C for 30 s; annealing at 50°C for 40 s; extension at 72°C for 50 s for 35 cycles then final extension at 72°C for 5 min. The products were determined by electrophoresis on 1% agarose gel at 120V/25 min and viewed on an ultra violet trans-illuminator (PREP ONE^{TM} Sapphire, EmbiTec) for a 120 bp product size.

Amplification of seb genes for S. aureus strains

The *seb* genes from the suspected enterotoxigenic *S. aureus* isolates were amplified using the *seb* primers (Table 1) on a DNA thermal cycler (Gene^{Amp} PCR system) ABI 9700 Applied Biosystems, SA) at a final volume of 40 µl for 35 cycles. The PCR mix included: 20 X2 Dream Taq Master Mix supplied by Inqaba Biotechnological Industries Ltd Pretoria, SA, the primers at a concentration of 0.4 µM each and 50 ng of the extracted DNA template. The PCR conditions were as follows: Initial denaturation at 95°C for 5 min; denaturation at 95°C for 30 s; annealing at 48°C for 30 s; extension, 72°C for 30 s for 35 cycles and final extension, 72°C for 5 min. The product was separated by electrophoresis on a 1% agarose gel at 120V/25 min and visualized on a UV trans-illuminator (PREP ONE^{TM} Sapphire, EmbiTec) for a 409 bp product size.

Antimicrobial susceptibility tests for isolated strains of *S. aureus* in ready-to-eat edible larvae and land snail meats

A Kirby-Bauer method as described by Cheesbrough (2010) was employed to test the antibiotic susceptibility pattern of confirmed *Staphylococcus* spp. Eleven commercially used antibiotics (Rapid labs, UK and Oxoid, Bastingstoke, UK) were employed; namely: amoxycillin/clavulanate or augmentin (AUG) 30 µg, cefoxitin (FOX) 30 µg, ceftazidime (CAZ) 30 µg, ceftriaxone (CTR) 30 µg, cefuroxime (CRX) 30 µg, cloxacillin (CXC) 5 µg, gentamicin (GEN) 10 µg, erythromycin (ERY) 5 µg, ofloxacin (OFL) 5 µg, oxacillin (OX) 1 µg and vancomycin (VA) 30 µg. The tests were performed by standard disk diffusion technique on Mueller Hinton agar (TM, Rajasthan, India) with the turbidity standard (0.5 McFarland). The plates were incubated aerobically at 35°C for 16 to 18 h. After

Table 2. Percentage mean proximate composition values of ready-to-eat roasted edible larvae (APW) and land snail meats.

Sample	Ash	Moisture	Crude lipid	Crude protein	Crude fibre	Carbohydrate
Roasted edible larvae	3.40±0.08	13.47±0.16	18.90±0.27	26.12±0.14	5.12±0.12	32.99±0.51
Land snail meat	1.11±0.02	59.60±0.65	3.88±0.06	13.61±0.22	2.01±0.02	19.79±0.46

APW =African palm weevil. Values with different superscripts are statistically significant (P = 0.05).

Table 3. Total viable bacterial counts in RTE land snail meat samples from the four different locations.

Sample	Sample location/Mean count (log_{10} CFU/g)			
	Mbiama	Choba-Emohua	Checkpoint (Ahoada)	Opokuma
1	6.39 ± 0.00^{ef}	7.12 ± 0.00^{f}	6.19 ± 0.00^{d}	6.09 ± 0.00^{c}
2	6.40 ± 0.00^{f}	6.95 ± 0.00^{e}	6.13 ± 0.00^{c}	6.13 ± 0.00^{c}
3	6.28 ± 0.00^{c}	6.06 ± 0.00^{b}	6.98 ± 0.00^{f}	6.01 ± 0.00^{b}
4	7.03 ± 0.00^{h}	6.04 ± 0.00^{b}	5.80 ± 0.00^{b}	6.91 ± 0.00^{e}
5	6.02 ± 0.00^{a}	5.94 ± 0.00^{a}	7.64 ± 0.00^{h}	7.16 ± 0.00^{f}
6	6.33 ± 0.00^{d}	6.03 ± 0.00^{b}	7.06 ± 0.00^{g}	5.97 ± 0.00^{b}
7	6.36 ± 0.00^{de}	6.34 ± 0.00^{c}	5.72 ± 0.01^{a}	5.65 ± 0.02^{a}
8	6.06 ± 0.01^{b}	5.95 ± 0.00^{a}	7.02 ± 0.01^{fg}	5.68 ± 0.00^{a}
9	6.98 ± 0.00^{g}	6.31 ± 0.00^{c}	6.66 ± 0.01^{e}	6.72 ± 0.01^{d}
10	7.02 ± 0.00^{h}	6.48 ± 0.01^{d}	6.16 ± 0.01^{cd}	7.27 ± 0.01^{g}

[a]Mean with different superscripts under each sample location are not equal (P<0.05). Means were compared across columns and mean with the same superscripts are equal. Mean counts ±SE (standard error).

incubation, test plates were examined for confluent growth and the diameter of each zone of inhibition was measured in millimetre (mm). Each inhibition zones diameter was interpreted using interpretive charts from WHO (CLSI, 2015).

Statistical analysis

The data obtained were analysed using descriptive statistics; one-way ANOVA and post Hoc Tukey test for significance P< 0.05. Proximate values of both food samples were in triplicates and analysed using Student T Test (p<0.05) to show their statistical significances. Some data were expressed in percentages, interpreted with tables using Microsoft Excel 2007.

RESULTS

Proximate values of ready-to-eat edible larvae and land snail meats

The mean proximate compositions of both ready-to-eat foods showed great differences between the nutritive values of edible larvae and snail meats (Table 2). The values of crude protein (26.12%), crude lipid (18.90%), fibre (5.12%), ash (3.40%) and total carbohydrate (32.99%) for edible larvae were higher compared to crude protein content (13.61%), lipid content (3.88%), fibre (2.01%), ash (1.11%) and total carbohydrate (19.79%) for snail meats with the exception of moisture content of snail meats (59.60%) which was much higher than edible larvae moisture content (13.47%).

Total viable bacterial counts of ready-to-eat land snail meats and edible larvae

The mean total viable bacterial count (TVC) of RTE snail meats is shown in Table 3. The result showed that TVC (log_{10} cfu/g) in Ahoada was the highest (5.72±0.01 to 7.64±0.0), followed by Opokuma (5.65±0.02 to 7.27±0.1), Choba-Emohua (5.94±0.01 to 7.12±0.0) and the lowest was Mbiama (6.02±0.0 to 7.03±0.0). Table 4 shows the mean TVC of the RTE edible larvae. The TVC (log CFU/g) showed that Opokuma had the highest count (6.72±0.01 to 9.01±0.01), followed by Tombia (6.29±0.0 to 8.43±0.0), Mbiama (5.90±0.03 to 8.26±0.0) while Ekeki had the lowest count (5.60±0.05 to7.47±0.0).

Total staphylococcal counts of the ready-to-eat land snail meats and edible larvae

The mean staphylococcal count of RTE snail meats for

Table 4. Total viable bacterial counts in RTE edible larvae from the four different locations.

Sample	Sample location/Mean count (log_{10}CFU/g)			
	Ekeki	Tombia	Mbiama	Opokuma
1	5.60 ± 0.05^{a}	7.58 ± 0.05^{g}	7.41 ± 0.00^{c}	9.01 ± 0.01^{j}
2	6.54 ± 0.00^{bc}	8.43 ± 0.00^{i}	7.45 ± 0.00^{c}	8.97 ± 0.00^{i}
3	7.0 ± 0.57^{bcd}	7.41 ± 0.00^{e}	8.11 ± 0.00^{d}	7.43 ± 0.00^{e}
4	6.58 ± 0.00^{bcd}	6.36 ± 0.00^{b}	8.26 ± 0.00^{d}	7.19 ± 0.00^{c}
5	7.36 ± 0.01^{bcd}	6.47 ± 0.00^{c}	7.43 ± 0.00^{c}	7.95 ± 0.00^{h}
6	7.47 ± 0.00^{d}	7.25 ± 0.00^{d}	6.32 ± 0.00^{b}	7.75 ± 0.00^{f}
7	6.59 ± 0.00^{bcd}	7.46 ± 0.00^{f}	6.27 ± 0.00^{b}	7.83 ± 0.00^{g}
8	7.46 ± 0.00^{d}	6.29 ± 0.00^{a}	5.98 ± 0.00^{a}	7.23 ± 0.00^{d}
9	7.43 ± 0.00^{cd}	8.38 ± 0.00^{h}	5.90 ± 0.03^{a}	7.05 ± 0.00^{b}
10	6.51 ± 0.01^{ab}	7.42 ± 0.05^{e}	6.31 ± 0.00^{b}	6.72 ± 0.01^{a}

[a]Mean with different superscripts under each sample location are not equal (P=0.05). Means were compared across columns and mean with the same superscripts are equal. Mean counts ±SE (standard error).

Table 5. Total staphylococcal counts in RTE land snail meat samples from four (4) different locations.

Sample	Sample location/Mean count (log_{10} CFU/g)			
	Mbiama	Choba-Emohua	Checkpoint (Ahoada)	Opokuma
1	6.05 ± 0.00^{b}	5.69 ± 0.05^{e}	5.53 ± 0.01^{b}	4.56 ± 0.00^{b}
2	5.90 ± 0.05^{b}	5.76 ± 0.00^{e}	5.70 ± 0.05^{b}	5.68 ± 0.00^{cd}
3	3.00 ± 0.57^{a}	5.91 ± 0.01^{f}	3.08 ± 0.00^{a}	5.38 ± 0.01^{bc}
4	5.93 ± 0.00^{b}	5.74 ± 0.00^{e}	5.27 ± 0.00^{b}	5.61 ± 0.00^{c}
5	3.18 ± 0.01^{a}	5.05 ± 0.00^{c}	5.60 ± 0.05^{b}	3.44 ± 0.00^{a}
6	2.78 ± 0.00^{a}	5.34 ± 0.01^{d}	3.60 ± 0.05^{a}	3.00 ± 0.57^{a}
7	3.26 ± 0.00^{a}	0.00 ± 0.00^{a}	3.00 ± 0.57^{a}	3.11 ± 0.00^{a}
8	5.66 ± 0.00^{b}	5.30 ± 0.06^{d}	3.08 ± 0.01^{a}	3.18 ± 0.00^{a}
9	3.20 ± 0.05^{a}	0.00 ± 0.00^{a}	3.85 ± 0.01^{a}	6.54 ± 0.00^{c}
10	5.79 ± 0.00^{b}	3.60 ± 0.05^{b}	5.91 ± 0.00^{b}	6.01 ± 0.01^{cd}

[a]Means with different superscripts under each sample location are not equal (p= 0.05). Means were compared across columns and means with the same superscripts are equal. Mean counts ± SE (standard error).

Mbiama, Choba-Emohua, Ahoada and Opokuma is shown in Table 5. Staphylococcal counts in log CFU/g were the highest in Opokuma (3.00±0.57 to 6.54±0.0) and the lowest were Choba-Emohua and Ahoada (3.60±0.05 to 5.91±0.01) and (3.00±0.57 to 5.91±0.0), respectively. Means within column with the same superscript are not significantly different (p=0.05). The mean total staphylococcal counts for edible larvae were the highest in Opokuma (4.51±0.0 to 8.62±0.01) log CFU/g and Ekeki had the lowest counts (3.70±0.1 to 6.76±0.0) log CFU/g, while Tombia and Mbiama staphylococcal counts (log_{10} CFU/g) ranged from (3.00±0.05 to7.45±0.0) and (3.28±0.0 to7.08±0.0), respectively (Table 6).

Interpretation of sanitary quality of the ready-to-eat foods edible larvae and land snail meats

In Table 7, the sanitary quality of the ready-to-eat food samples were interpreted with consideration to microbiological guidelines for ready-to-eat foods; category 5 (cooked foods chilled but with some handling prior to sale or consumption). All 80 analysed samples of both RTE edible larvae and snail meats revealed that 58.75% (47 of 80) of both samples were below the acceptable limit (10^5 to<10^7) CFU/g while 41.25% (33 of 80) of the samples were unsatisfactory (≥10^7 CFU/g). Total staphylococcal counts of the samples showed that 7. 5% (6 of the 80) were within acceptable limit (< 20

Table 6. Total staphylococcal counts in RTE roasted edible larvae from four (4) different locations.

Sample	Sample location/Mean count (log_{10} CFU/g)			
	Ekeki	Tombia	Mbiama	Opokuma
1	5.53 ± 0.01^{d}	7.40 ± 0.05^{d}	7.08 ± 0.00^{g}	8.56 ± 0.00^{h}
2	6.03 ± 0.00^{f}	7.45 ± 0.00^{d}	0.00 ± 0.00^{a}	8.62 ± 0.01^{i}
3	5.83 ± 0.00^{e}	6.15 ± 0.00^{b}	3.28 ± 0.00^{b}	6.68 ± 0.01^{de}
4	3.95 ± 0.00^{c}	6.89 ± 0.00^{bcd}	6.27 ± 0.00^{e}	6.72 ± 0.01^{e}
5	6.20± 0.05^{g}	7.09 ± 0.00^{cd}	6.30 ± 0.00^{f}	7.56 ± 0.01^{g}
6	0.00 ± 0.00^{a}	6.26 ± 0.01^{bc}	3.30 ± 0.00^{b}	6.93 ± 0.02^{f}
7	0.00 ± 0.00^{a}	7.41 ± 0.00^{d}	6.15 ± 0.00^{d}	6.65 ± 0.00^{d}
8	6.76 ± 0.00^{h}	3.26 ± 0.00^{a}	5.28 ± 0.00^{c}	6.48 ± 0.00^{c}
9	3.70 ± 0.10^{b}	3.00 ± 0.570^{a}	0.00 ± 0.00^{a}	4.62 ± 0.01^{b}
10	5.64 ± 0.01^{d}	3.18 ± 0.00^{a}	6.26 ± 0.01^{e}	4.51 ± 0.00^{a}

[a]Means with different superscripts under each sample location are not equal (p= 0.05). Means were compared across columns and means with the same superscripts are equal. Mean counts ± SE (standard error).

CFU/g), 27. 50% (22 of 80) at borderline limit (20 to ≤10^4) CFU/g and 65.00% (52 of 80) were unsatisfactory (>10^4 CFU/g). The RTE edible larvae from Opokuma and Tombia were the most unsatisfactory for consumption whereas the RTE snail meats samples were mostly at the borderline (10^5 to<10^7 CFU/g) (Health Protection Agency, 2009).

Detection of *Staphylococcus* spp. in the RTE roasted edible larvae and land snail meats

Out of the total of 80 samples (40 each) of the RTE edible larvae and snail meats analyzed, 74 (92.5%) and 47 (58.75%) of both RTE roasted edible larvae and land snail meats were positive for *Staphylococcus* spp. and *S. aureus*, respectively.

Molecular characterization of *Staphylococcus* spp. isolated from both ready-to-eat foods

Detection of enterotoxins A and B genes in the isolated Staphylococcus using PCR

Gel electrophoresis of *seb* genes from the isolated *Staphylococcus* strains: The agarose gel electrophoresis of amplified *seb* genes obtained when the DNA of *S. aureus* isolates were subjected to PCR using *seb* gene primers is as shown in Figure 3. A product size observed was 409 bp for *seb* genes. Lane L represents the molecular ladder lanes; N5, N7, and N15 showed positive on the *seb* gene bands.

Gel electrophoresis of *sea* genes from isolated *Staphylococcus* strains: The agarose gel electrophoresis of amplified *sea* genes obtained when the DNA of *Staphylococcus* isolates were subjected to PCR using *sea* gene primers is as shown in Figure 4. A product size observed was 120 bp for *sea* genes. Lane L represents the 100 bp molecular ladder lanes; N3, N5, N6, N7, N12, N14 and N15 showed positive on the sea gene bands.

Distribution of staphylococcal enterotoxins *sea* and *seb* genes in both ready-to-eat foods: In Table 8, *Staphylococcus* strains in both ready-to-eat foods produced *sea* and/or *seb*. SISA and OPSMI codes for *S. aureus* O326 and *S. warneri* JRT4, respectively harboured only sea virulent gene each. Also, 3/8 of *S. aureus* strains (with isolate codes ES3MS2a, SIMS3a and TS2Ma1) isolated from RTE edible larvae harboured both sea and *seb* virulent genes and isolated from Mbiama, Ekeki and Tombia, respectively. Other 2 strains (represented as S1MS2 and TS2MS1a) isolated from edible larvae produced only sea virulent genes and were obtained from Ekeki and Tombia, respectively. No *seb* virulent gene occurred singly in the 13 *S. aureus* strains.

Antibiotic resistance pattern of tested *Staphylococcus* spp. in both ready-to-eat foods: The *S. aureus* strains from both RTE edible larvae and snail meats were tested with 11 antibiotics. Gentamicin, ofloxacin and erythromycin were most active against all *S. aureus* strains at 100%. The resistance to ceftazidime,

Table 7. Interpretation of total viable bacteria counts and total staphylococcal counts of ready-to-eat edible larvae and land snail meats with microbiological guidelines for ready-to-eat foods – category 5.

RTE food sample	Sampling area	No. of samples (n)	TCV CFU/g			TSC CFU/g		
			Satisfactory	Borderline	Unsatisfactory	Satisfactory	Borderline	Unsatisfactory
		n=80	$<10^5$	10^5-$<10^7$	$\geq10^7$	<20	20-$\leq10^4$	$>10^4$
Roasted edible larvae	Mbiama	10	-	5	5	2	2	6
	Ekeki	10	-	5	5	2	2	6
	Tombia	10	-	3	7	-	3	7
	Opokuma	10	-	1	9	-	-	10
Snail meats	Mbiama	10	-	8	2	-	5	5
	Choba-Emohua	10	-	9	1	2	1	7
	Ahoada	10	-	7	3	-	5	5
	Opokuma	10	-	9	1	-	4	6
Total				47 (58.75%)	33 (41.25%)	6 (7.50%)	22 (27.50%)	52 (65.00%)

TVC= Total viable count; TSC= total staphylococcal count. Food category 5= cooked foods chilled but with some handling prior to sale or consumption.

augmentin (amoxycillin-clavulanate) and cloxacillin was highest (100%). They showed varying resistance against ceftriaxone (57.14%), cefuroxime (28.57%), vancomycin (42.86%), oxacillin (42.86%) and cefoxitin (42.86%) (Figure 5).

Multiple-antibiotic resistance profile of *Staphylococcus* spp. isolated from the RTE samples: *Staphylococcus* spp. showed varying multiple resistances to antibiotics as presented in Table 9. *Staphylococcus warneri* JRT4 obtained from Opokuma had the highest percentage resistance of 63.6% to multiple antibiotics, followed by *S. aureus* O326 obtained from Choba-Emohua with 54.5%. *S. aureus* O326 and *S. aureus* P3-1 isolated from Mbiama and Ekeki, respectively showed the least multiple resistances of 27.3%.

DISCUSSION

In this study, proximate composition, microbiological qualities of the ready-to-eat edible larvae (*R. phoenicis*) and land snail (*A. marginata*) meats, presence of virulent genes and antibiotic sensitivities of *S. aureus* were investigated.

The proximate compositions of the ready-to-eat foods indicated that both RTE foods are sufficient in nutrients and edible larvae have more nutritional values compared to land snail meats. A number of authors have reported that edible larvae are very excellent sources of protein (Banjo et al., 2006; Opara et al., 2012; Amadi et al., 2014; Ebenebe and Okpoko, 2015) in comparison with proteins in chicken (24.96%), pork (16.57%), goat meat (20.14%) and beef (18.81%) (Afolabi et al., 2017). The findings of this study revealed that the protein content of RTE snail meats (13.6%) was less than the protein contents of 3 diverse kinds of unprocessed land snail meats ranging from 15.44 to 72.64% (Adegoke et al., 2010). The proximate value of lipid in this study is comparable to19.54% reported for the same kind of larvae by Okaraonye and Ikewuchi (2008). The lipid content of pork (36.87%) showed great disparity to lipid contents of edible larvae and snail meats (Afolabi et al., 2017). The lipid value (3.88%) for RTE snail meats in the current study is more than the crude lipid contents range (1.15 to 1.52 %) reported by Adegoke et al., (2010). Akinnusi (2002) stated that the low content of fat and low cholesterol level make snail meat a remedy for vascular diseases such as heart attack, cardiac arrest, hypertension, stroke, high blood pressure and other fat related ailments. Contrary to reports by Opara et al., (2012) that edible larvae are a poor energy giving foods, the carbohydrate content of edible larvae in this study was high (32.99%). The moisture content of these foods evaluated reveals their

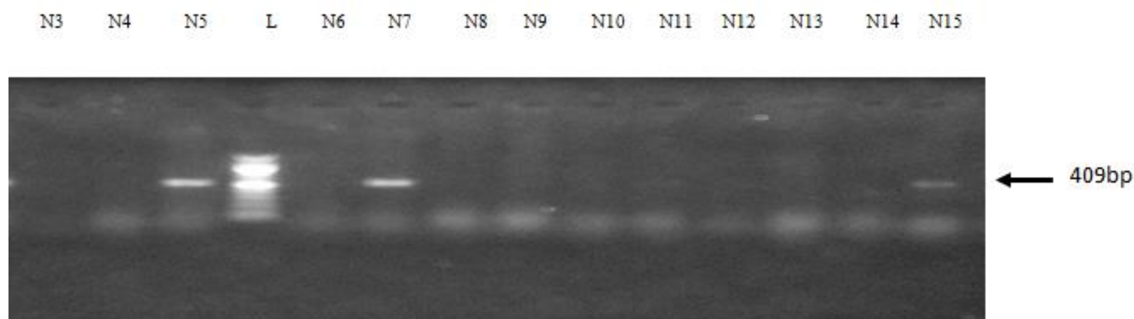

Figure 3. The agarose gel electrophoresis of the amplified *seb* genes in the *Staphylococcus*. Lanes N5, N7, and N15 showed the *seb* bands (409bp) while lane L represents the 100bp molecular ladder. N3= S1SA, N4=TS2MS1b, N5=ES3MS2a, N6=OPSM1, N7=S1MS3a, N8=S3A2, N9=S2SA, N10=S3A, N11=S1MP1, N12=S1MS2, N13=ES3MS1b, N14= TS2MS1a, N15= TS2Ma1

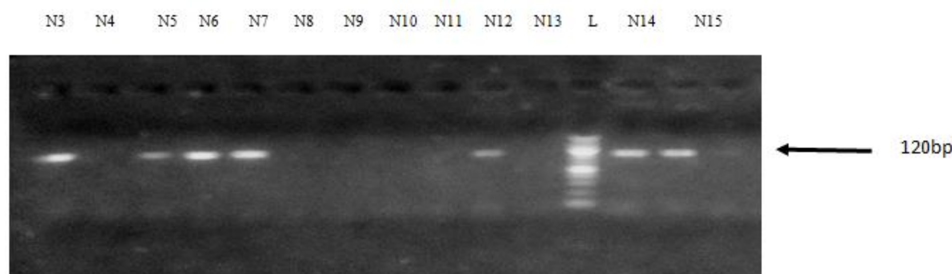

Figure 4. The agarose gel electrophoresis of the amplified *sea* genes in *Staphylococcus.* Lanes N3, N5, N6, N7, N12, N14, N15 showing the *sea* gene bands while lane L represents the 100bp molecular ladder. N3= S1SA, N4=TS2MS1b, N5=ES3MS2a, N6=OPSM1, N7=S1MS3a, N8=S3A2, N9=S2SA, N10=S3A, N11=S1MP1, N12=S1MS2, N13=ES3MS1b, N14= TS2MS1a, N15= TS2Ma1.

Table 8. Distribution of staphylococcal enterotoxins (*sea* and *seb* genes) in the ready-to-eat foods in different locations.

Isolate code	strains	Virulence genes detected	Source of isolation	Location
S1SA	*S. aureus* O326	*sea*	Snail meat	Mbiama
OPSM1	*S. warneri* JRT4	*sea*		Opokuma
ES3MS2a	*S. aureus* O326	*sea, seb*	Edible larvae	Mbiama
S1MS3a	*S. aureus* O326	*sea, seb*		Ekeki
S1MS2	*S. aureus* P3-1	*sea*		Ekeki
TS2MS1a	*S. aureus* O326	*sea*		Tombia
TS2Ma1	*S. aureus* O326	*sea, seb*		Tombia

stability and susceptibility to contamination by organisms (Uraih and Izuagbe, 1990). The low fibre content of RTE snail meats implies that they can serve as roughage. Edible larvae are adequate source of diet roughage for bowel peristalsis (Okaraonye and Ikewuchi, 2008). The nutritive values of both foods show they are considerate reserves of protein, lipid, carbohydrate and moisture.

Most of the food samples had TVC exceeding 10^5 CFU/g as has been reported by other authors in Nigeria, Congo and Sri Lanka (Ekrakene and Igeleke, 2007;

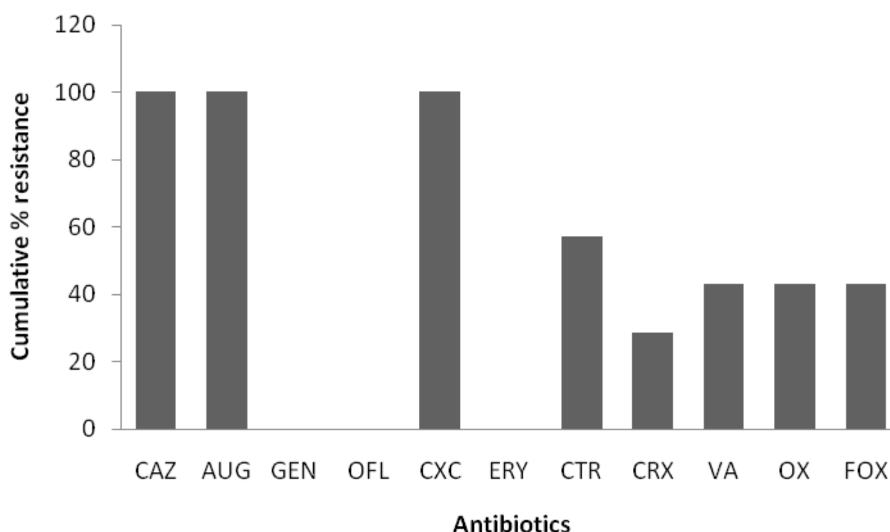

Figure 5. Antibiotic resistance pattern of pathogenic *Staphylococcus aureus* strains isolated from both ready-to-eat edible larvae and land snail meats. CAZ = Ceftazidime, AUG = augumentin, GEN = gentamicin, OFL = ofloxacin, CXC= cloxacillin, ERY = erythromycin, CTR = ceftriaxone, CRX = cefuroxime, VA = vancomycin, OX=oxacillin and FOX = cefoxitin.

Table 9. Multiple-antibiotic resistance profile of strains of *Staphylococcus aureus* and *Staphylococcus warneri* isolated from both ready-to-eat foods.

Isolate code	Name of strains	Antibiotics resistant to	Occurrence of resistance	Resistance % of strains
S1SA	*S. aureus* O326	CAZ, AUG, CXC	3	27.3
OPSM1	*S. warneri* JRT4	CAZ,AUG,CXC,CTR,OX,VA,FOX	7	63.6
ES3MS2a	*S. aureus* O326	CAZ, AUG, CXC, CTR	4	36.4
S1MS3a	*S. aureus* O326	CAZ, AUG, CXC, CTR, FOX	5	45.5
S1MS2	*S. aureus* P3-1	CAZ, AUG.CXC	3	27.3
TS2MS1a	*S. aureus* O326	CAZ, AUG, CXC, OX	4	36.4
TS2Ma1	*S. aureus* O326	CAZ,AUG,CXC,VA,OX.FOX	6	54.5

CAZ = Ceftazidime, AUG = augmentin, GEN = gentamicin, OFL = ofloxacin, CXC= cloxacillin, ERY = erythromycin, CTR = ceftriaxone, CRX = cefuroxime, VA = vancomycin, OX=oxacillin, FOX = cefoxitin.

Ebenebe and Okpoko, 2015; Makelele et al., 2015; Wimalasekara, 2016). The TVC was the highest in RTE snail meats from Ahoada (5.72 to 7.64 $\log_{10}$ CFU/g), while for RTE edible larvae, Opokuma had the highest counts (6.72 to 9.01 $\log_{10}$ CFU/g). Total presumptive staphylococcal counts in snail meats and edible larvae were both highest in Opokuma having counts ranging from 3.0 to 6.54 $\log_{10}$ CFU/g and 4.51 to 8.62 $\log_{10}$ CFU/g, respectively. In line with the TVC for RTE snail meats, Nyoagbe et al. (2016) reported TVC in land snail species ranging from 6.00 to 8.00 $\log_{10}$ CFU/g while the TSC range from 2.00 to 7.00 $\log_{10}$ CFU/g. The enormous degree of contamination in these ready-to-eat foods may not be unrelated to the processing environment, initial microbial loads, extent of exposure at selling points, and food handling by food vendors. Staphylococci counts in cooked foods suggest improper and repeated food handling after food processing. High counts of *Staphylococcus* spp. in cooked foods renders them unfit for man consumption.

The results of virulence gene detection revealed an 8.64% (7 of 81) harbours *sea* genes, 6 *S. aureus* and *S. warneri* strains while 3.70% (3 of 81) harbours the *sea* genes, 3 *S. aureus* strains. This finding is not comparable to the research findings carried out in Gorgan city of Iran (Kamarehei et al., 2013) which reported that out of 170 *S. aureus* isolates, 60.6% contained *sea* genes while 27.1% carried *seb* genes, but followed the same trend with the

sea genes dominating. Leke et al. (2017) also reported that 24.1% *S. aureus* isolated from breast milk, had *sea* gene. The *sea* gene is the most common toxin associated with staphylococcal food poisoning and *seb* is also linked to food poisoning as well as an inhaled bio-weapon (Balaban and Rasooly, 2000; Pinchuk et al., 2010). The study further revealed that the *S. aureus* produced either *sea* and/or *seb* genes. *S. aureus* P3-1 produced only *sea* genes whereas only *S. aureus* O326 obtained from both RTE foods produced *sea* and/or *seb* genes. *S. aureus* O326 was the most distributed strain in the foods across the sampled locations. *S. aureus* O326 and P3-1 present in the edible larvae at Ekeki, while *S. warneri* JRT4 in Opokuma. The virulent genes presence demonstrated the potential toxigenicity and pathogencity of the *Staphylococcus* isolates. The *sea* and *seb* are classical types of SEs and influence emetic activity. The *sea* genes were isolated both in edible larvae and snail meats while *seb*-carrying strains were only common in edible larvae. This occurrence is possibly caused by epidemiological factors like type of strains and environment. Delayed processing, inadequate refrigerating, poor personal cleanliness and post-process contamination are related to the growth of *S. aureus* strains harbouring SE and ET genes (Leke et al., 2017).

Previous reports confirmed the enterotoxigenicity of other *Staphylococcus* spp. other than *S. aureus* (Jay, 1992). In this study, *S. warneri* JRT4 harboured staphylococcal enterotoxins *sea* genes and biochemically was positive to coagulase. According to Becker et al., (2001), few coagulase positive *Staphylococcus intermedius* strains harbour SEs present in food.

Antimicrobial resistance from foods and water sources are of global concern (Kumar et al., 2005). There were varying degrees of resistance of the *Staphylococcus* strains to the commercial antibiotics. The virulent genes bearing *Staphylococcus* were 100% resistant to augmentin, ceftazidime and cloxacillin but showed varying resistance against ceftriaxone (57.14%), cefuroxime (28.57%), vancomycin (42.86%), oxacillin (42.86%) and cefoxitin (42.86%). Overall, they were 100% sensitive to gentamicin, ofloxacin and erythromycin. High degree of sensitivity of different organisms to ofloxacin and gentamicin has been previously reported (Okonko et al., 2008; Mordi and Momoh, 2009; Umofia, 2012; Afolabi et al., 2017). Agbo et al. (2016) reported that *S. aureus* isolated from street foods in Calabar had varying resistance to ciprofloxacin (20%), gentamicin (10%), and levofloxacin (5%). Kumar et al. (2009) reported higher resistance of *S. aureus* against erythromycin (87.5%), while Achi and Madubuike (2007) reported lesser resistance against erythromycin (0.83%) which is comparable to the findings of this study. According to Harakeh et al. (2005) and Guven et al., (2010), meals prepared in the street provide a suitable culture medium for *S. aureus* strains impervious to various antibiotics and transmitted to people via street foods that are contaminated. *S. aureus* are usually regarded to be resistant to antibiotic therapy, as a result of exopolysaccharide obstruction and their sites inside micro abscesses which reduce the actions of drugs (Gundocan et al., 2006). Food handlers and the surrounding that harbour antibiotic resistant *S. aureus* strains transfer their resistant genes to the sustenance likely because of their poor cleanliness and sanitation or the misuse of antibiotic administrations. *S. aureus* O326 and *S. warneri* JRT4 isolated from RTE snail meat from Choba and Opokuma, respectively showed highest multiple resistances to the selected antibiotics confirming earlier report by Waters et al. (2011) that multidrug resistance *S. aureus* occurs often.

In this finding, ofloxacin, a second era quinolones, erythromycin and gentamicin, an aminoglycosides are the most dynamic broad spectrum antibacterial agents to combat ailments brought about by the *Staphylococcus*.

Conclusion

The findings of this investigation demonstrated that RTE foods examined are excellent sources of essential nutrients, with edible larvae having more nutritional benefits compared to land snail meats. The study showed that 41.25% samples tested had total viable above the acceptable microbiological limit (≥10^7 CFU/g) for ready-to-eat vended foods. *Staphylococcus* spp. carried the *sea* and/or *seb* virulence genes affirming their ability to initiate disease conditions for commuters who patronize these vendors. The strains of *S. aureus* and *S. warneri* isolated from the RTE edible larvae and snail meats showed various dimensions of resistance pattern to the commercial antibiotics utilized in global reports, hence the requirement for a superior and realistic approach to ensure road side food vendors comply with standard food safety practices.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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