Nig. J. Biotech. Vol. 40(2): 90-98 (Dec 2023)
ISSN: 0189 1731
Available online at
http://www.ajol.info/index.php/njb/index
and https://bsn.org.ng
DOI: https://dx.doi.org/10.4314/njb.v40i2.10

NIGERIAN JOURNAL OF BIOTECHNOLOGY

Screening for Virulent and Antibiotic Resistant *Escherichia coli* in Raw and Ready-to-Eat Snails (*Arachatina marginata*) Vended in Selected Markets in Port Harcourt, Nigeria

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Abstract

This aim of this study was to screen for virulent and antibiotic resistant *Escherichia coli* in raw and ready-to-eat snails (*Archachatina marginata*) vended in selected markets within Port Harcourt, Nigeria. Proximate composition, isolation, identification and presence of virulent genes were done using standard methods. Raw snails from Choba market had *Escherichia coli* count ranging from $1.8x10^4$ to $8.2x10^5$ CFU/g, while the ready-to-eat samples were not contaminated with *E. coli*. Raw snails from Rumuokoro market had *Escherichia* counts ranging from $4.2 x10^4$ to $6.9 x10^6$ CFU/g while two of the ready-to-eat samples had counts of $3.602 x 10^3$ CFU/g and $3.556 x 10^3$ CFU/g. Raw snails from Oyigbo market had *Escherichia* counts ranging from $4.653 x 10^3$ CFU/g to $5.579 x 10^3$ CFU/g while one ready-to-eat sample had *Escherichia*. Antimicrobial susceptibility testing showed that 100% of *Escherichia* were resistant to cefuroxime, augmentin, cefixime and ceftazidime. Of the three-virulence genes (*eae*, *ast* and *aggR*) screened for in this study only *aggR* was detected in one *Escherichia* isolate. Proper blanching and heating should be employed during preparations of snails to curtail food poisoning.

Keywords: Antibiotic resistance, *Archachatina marginata Escherichia coli*, Virulence gene,
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Introduction

Giant African land snails (*Archachatina marginate*) are known to play valuable roles in the nutritional life and culture of rural dwellers, serving as a very good delicacy among villagers, urban dwellers and among international hoteliers (Abiona et. al., 2013). Most people in Nigeria use snail as a replacement for beef in locally prepared delicacies as the meat competes favourably with poultry egg and fish in essential amino acids and digestible protein (Imevbore, 1990). Azage and Kibret (2017) described the protein composition of snail meat as not only consisting of amino acids but excellent and the most essential ones the body requires. They are a good source of lysine, leucine, arginine and tryptophan and contains high level of iron, calcium, and phosphorus, and are said to contain almost all the amino acids needed by humans (Amusan & Omidiji, 1998; Nyoagbe et. al., 2016) but low in sodium, fat and cholesterol (Efuntoye et al., 2011).

Snails are reared in environment that encourages microbial proliferation. Thus, a close association exists between microorganisms and snails because of their growing environment (Nyoagbe et. al., 2016; Daminabo et. al., 2020). Snail farms are always filthy with feces and decomposing materials as such, the high microbial load often found among snail population is not unexpected. This interaction with microorganisms makes them accumulate microorganisms which may be pathogenic from the smut found in their habitat (Fagbuaro, 2006). The microorganisms therefore continue to inhabit snail environment all through

their stages of development and be disseminated through the faeces and slimy fluid produced by the snails (Fagbuaro, 2006).

Evaluating the microbiological content of both raw and ready-to- eat snail meat is therefore, very necessary since they are potential carriers of disease-causing bacteria. Bacterial species that belong to the Enterobacteriaceae (*Citrobacter, Morganella, Klebsiella, Enterobacter, Escherichia coli, Pseudomonas, Proteus, Hafnia, Yersinia enterocolitica*), gram-positive *Staphylococcus* and *Listeria*; fungal species of the genera *Aspergillus, Chrysosporium, Fusarium, Rhizopus* and *Mucor* and parasites *such as Clonorchissinensis* and *Fasciola* species in snail-shell have all been documented (Cicero et. al., 2015).

Snails are known vector for the spread of enteric pathogens, including *E. coli* (Nwiyi & Amaechi, 2013). Though this enteric microorganism may reside in snails without causing harm to them, reports of the occurrence of pathogenic strains of *E. coli* from snails' sources and outbreaks of illness occurs increasingly in humans when they are eaten without proper cooking (Nwiyi & Amaechi, 2013; Nwuzo et. al., 2016).

Contamination of snails to be sold often occur because of the ways they are handled and exposed in the markets. However, raw snails are contaminated as they feed on filths which are sure site for microbial proliferation (Nyoagbe et. al., 2016). The inherent microbial load of ready-to-eat snail increases during exposure as they are not showcased at low temperatures that will retard microbial growth. A substantial population of microbial pathogens in meat products can survive the heat during cooking processes thereby given a form of protection.

Antibiotic resistant *E. coli* has become a problem in recent decades, as strains of bacteria exhibiting resistance to several antibiotics have become more common (Paterson & Bonomo, 2005; Perfeito et. al., 2007). Antibiotic-resistant *E. coli* may also pass on the genes responsible for resistance to other species of bacteria. Mixing of species in the intestines allows *E. coli* to allow horizontal transfer of plasmids. Thus, *E. coli* and the other enterobacteria are important reservoirs of transferable antibiotic resistance (Salyers et. al., 2004).

The acquisition of virulence and resistance genes increase the pathogenicity of microorganisms and the severity of infection with the possibility of therapy failure and the increase mortality rate. (Abd El-Baky et. al., 2020). Commensal *E. coli* may acquire virulence genes that may, in combination, result in intestinal and extra-intestinal *E. coli* infections (Abd El-Baky et. al., 2020). *Escherichia coli* produces bacteriocins such as colicins and microcins which are bactericidal peptides and considered as virulence factors in different *E. coli* strains or pathotypes (Micenková et. al., 2017).

Food-borne diseases are prevalent in developing nations and many of the culpable pathogens are present in snails (Adagbada et. al., 2011). This study aimed at investigating presence of virulent and antibiotic resistant *E. coli* in raw and ready-to-eat land snails (*Archachatina marginata*) vended in selected markets within Port Harcourt.

Materials and Methods

Collection of samples

Fifty (50) samples, twenty-five (25) samples each of both raw and ready-to-eat African land snails were sourced randomly from different vendors in Choba, Rumuokoro and Oyigbo Markets in Port Harcourt. They were transported in sterile container to Emadavistic Laboratory, Osaks House, East-West Road, Choba for analysis.

Proximate Analysis

Proximate analysis and the nutrient status were carried out on the raw and ready-to-eat snail meat to determine their nutritional status. Determination of moisture, crude protein, fat, ash, total available carbohydrate and crude fibre were done according to the guidelines of the Association of Official Analytical Chemists (AOAC, 2005).

Isolation of Bacteria

The culture media used include: Nutrient agar and Eosin Methylene blue agar. Ten grams (10 g) of the sample from different locations were added to 90 ml of peptone broth, swirled for few minutes followed by a ten-fold serial dilution. From the prepared dilutions, 0.1 ml of each of 10^{-4} and 10^{-5} dilutions were transferred onto the solidified media on Petri plates and was spread gently using sterile bent glass rod. The plates were incubated at 37^{o}C for 18-24 h and representative colonies with green metallic sheen were further purified on freshly prepared nutrient agar and stored on slants for confirmation and further studies.

Characterization and Identification of Isolates

The isolates were confirmed on the basis of their cultural morphology, physiological and biochemical properties (Gram's reaction, indole, methyl red, Voges-Proskauer and citrate) (Cheesbrough (2000).

DNA Extraction

DNA was extracted using the boiling method as described by Hitchins et. al. (2004). Cells were harvested by centrifuging overnight pure culture of *E. coli* isolates in 2 ml Eppendorf tubes for 2 min at 10,000 rpm. The supernatants were discarded. Pellets were resuspended in 100 µl of distilled water, boiled for 10 min in water bath, after which it was centrifuge at 10000 rpm. Supernatants were then transferred to fresh Eppendorf tubes and stored at -20^{o}C until further analysis.

Determination of Virulent Genes

Oligonucleotide primers for *aggR*, *eae* and *ast* virulence genes described by Bisi-Johnson et. al. (2011) was employed (Table 1). Polymerase chain reaction (PCR) was conducted in thermocycler (Mastercycle-Eppendorf, Vapour Product, Germany) in a volume of 25 µl 10xPCR buffer, 25 nM $MgCl_2$, 2.5 DNTPs each of appropriate 0.1 primer, 0.1µl Taq polymerase, 10 µl of appropriate DNA preparation and 13.4 µl sterile distilled water. Amplification following an initial denaturation at 94^{o}C for 5 min was performed in 35 cycles at 94 ^{o}C for 15s, 55^{o}C for 20 s and 72 ^{o}C for 30 s. A final extension was done for 7 min at 72 ^{o}C. An 8 µl aliquot of the PCR product mixed with a loading dye (10mM, EDTA, 10% glycerol, 0.015% bromo phenol dye and 0.017% SDS, made up to 100 ml) were checked using Portable Gel hood built in Blue LED (470 nm) by Royal Biotech/Biolympics,1.5% agarose gel at a constant voltage and 1X TBE for approximately 1 h. They were visualized by Ethidium bromide staining and photographed under ultraviolet light. The ladder used was 1kb base pair from thermo scientific.

Table 1: Primers sequences.

Target gene	**Primer Nucleotide Sequence** (5′-3′)	**Amplicon size (bp)**
aggR	F GTATACACAAAAGAAGGAAGC	256bp
	R ACAGAATCGTCAGCATCAGC	
eae	F ATGCTTAGTGCTGGTTTAGG	248bp
	R GCCTTCATCATTTCGCTTTC	
ast	F GCCATCAACACAGTATATCC	106bp
	R GAGTGACGGCTTTGTAGTCC	

Antibiotics Susceptibility Testing

Antibiotic sensitivity of all the confirmed isolates was performed by standard disk diffusion method according to Kirby-Bauer on Mueller-Hinton agar (Titan, Biotech Ltd, Indian) following the procedures as described in Bauer et. al. (1959). Eight commonly used antibiotics (µg/disc) viz. Amoxicillin-clavulanate or augmentin (AUG), gentamycin (GEN), nitrofurantoin (NIT), cefuroxime (CRX), ofloxacin (OFL), cefixime (CXM), ciprofloxacin (CPR) and ceftazidime (CAZ), Abtek, (UK) were tested. From an overnight culture of *E. coli,* 0.5 MacFarland turbidity standard bacterial suspension was prepared in sterile saline, from which 0.1 ml was inoculated and spread on Mueller Hinton agar. Thereafter, antibiotic discs were carefully and aseptically placed on the surface of the agar. The plates were incubated at 37^{o}C for 24 h. Zones of inhibition were measured in millimeter.

Results

Nutritional Composition of Raw and Ready-to-eat Snails

Proximate analysis done on representative raw and ready-to-eat snail samples showed varying percentages in nutritional composition. Raw snail had more moisture (78.33 %) compared to the ready-to-eat (66.67%) while in terms of crude protein, ready-to-eat snail meat had average of 12.03 % compared to the 10.01% recorded for raw snail meat. Likewise, ready-to-eat snails had a relatively higher carbohydrate content (11.61%) when compared to the raw sample (3.19%) (Table 2).

Table 2: Average Percentage Nutritional Composition of Raw and Ready-to-eat Snails Examined.

Parameter	Percentage (%) Composition	
	Raw Sample	Ready-to-eat Sample
Ash	2.67	3.13
Moisture Content	78.33	66.67
Crude Lipid	2.50	1.76
Crude Protein	10.01	12.03
Crude Fibre	3.20	4.80

Carbohydrate	3.29	11.61
Ash	2.67	3.13

Occurrence of *E. coli* in the Various Samples Examined

The number of *E. coli* isolated from the various samples obtained from different markets are as presented in Table 3. The results showed that all raw samples had *E. coli.* For the ready-to-eat snail samples, *E. coli* was detected in samples sourced from Rumuokoro and Oyigbo markets.

Table 3: Occurrence of *E. coli* in the Various Raw and Ready-to-eat Snail

Markets	No of samples collected	Raw Snail	Ready-to-eat Snail
		No. positive for *E. coli* (%)	No. positive for *E. coli* (%)
Choba	10	10 (100%)	0
Rumuokoro	10	10 (100%)	2(20%)
Oyigbo	5	5 (100%)	1(20%)
Total	25	25 (100%)	3 (12%)

The ten (10) samples of raw snail sourced from Choba markets had counts ranging from $1.8x10^4$ to $8.2x10^5$ CFU/g while the ready-to-eat samples were free of *E. coli* contamination (Figure 1).

The ten (10) samples of raw snail sourced from Rumuokoro markets had counts ranging from $4.2x10^4$ to $6.9x10^6$ CFU/g while the ready-to-eat samples were had counts ranging from $3.9x10^3$ to $3.5x10^3$CFU/g (Figure 2).

The Five (5) samples of raw snail sourced from Oyigbo market had counts ranging from $4.5x10^4$ to $3.8x10^5$ CFU/g while only one sample from the ready-to-eat samples was contaminated with *E. coli* ($3.9x10^4$ CFU/g) (Figure 3).

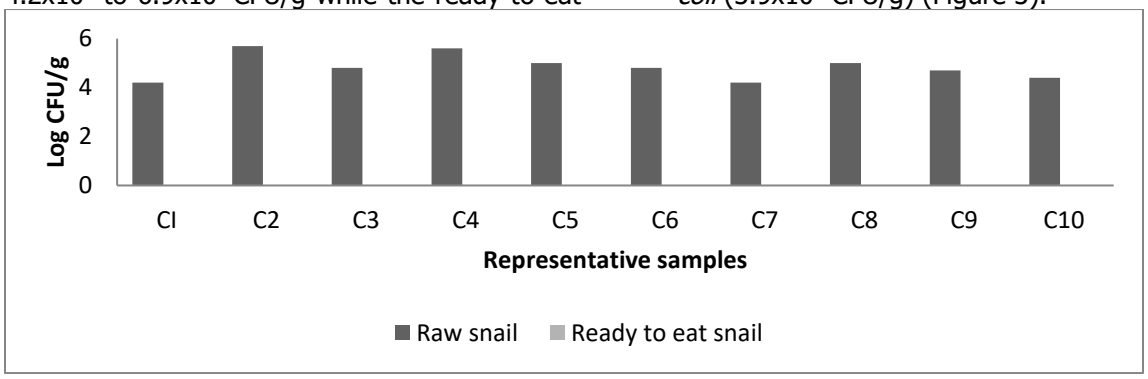

Fig 1: Occurrence of *Escherichia coli* in Raw and Ready-to-eat Snail Sampled from Choba Market

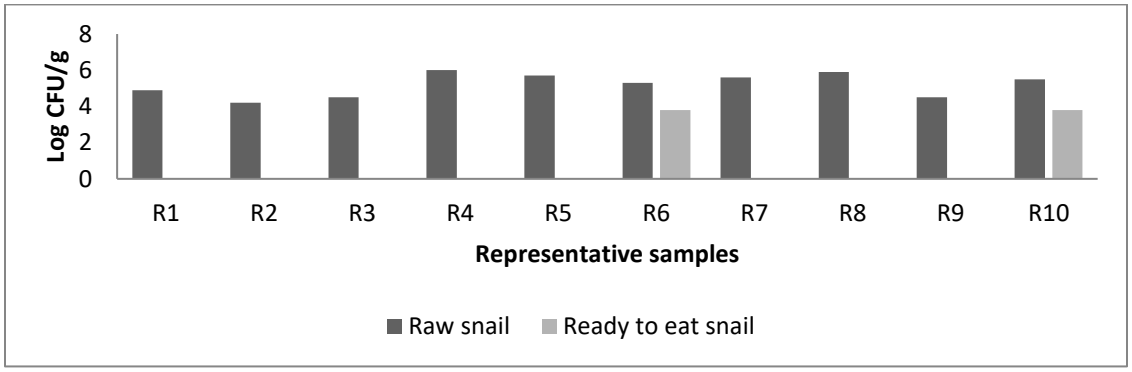

Fig 2: Occurrence of *Escherichia coli* in Raw and Ready-to-eat Snails Sampled from Rumuokoro Market

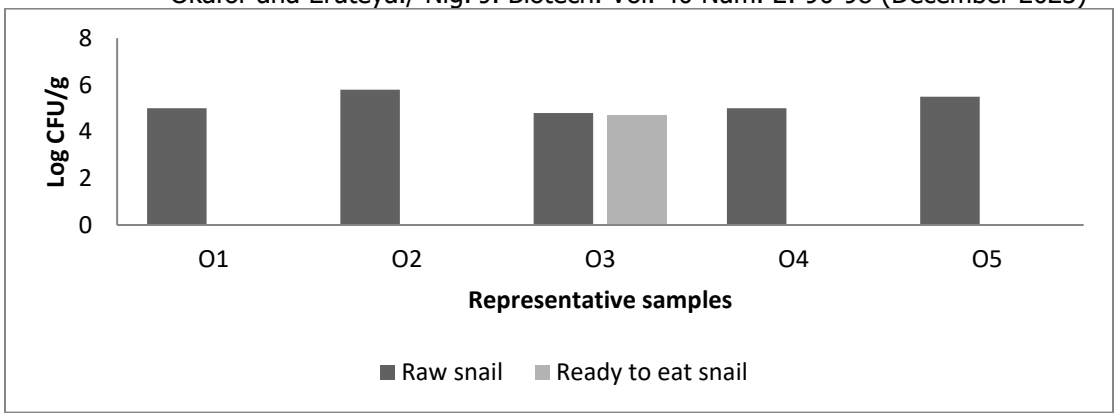

Fig 3: Occurrence of *Escherichia coli* in Raw and Ready-to-eat Snail Sampled from Oyigbo Market

Antibiotics Susceptibility of E. coli Isolated from Snail Meats

The antimicrobial susceptibility pattern of all *E. coli* isolated from both raw and ready-to-eat snails showed varying sensitivity to the different antibiotics. The *E. coli* isolates from the different snails showed 100 % susceptibility to augmentin, ciprofloxacin, cefixime and ofloxacin (Table 4). The results of the antibiotic test showed *E. coli* were 100% resistant to ciprofloxacin (CPR) and ofloxacin (OFL) across the different markets studied of the antibiotics tested. There were 100% (n=53) *E. coli* resistance to CRX, AUG, CAZ and CXM while 100% (n=53) susceptibility was recorded for CPR and OFL. However, varying sensitivity was recorded across the markets for *E. coli* against other antibiotics. Fifty-eight percent 58.5 % (n=31) of *E. coli* was sensitive to NIT with 32.1% (n=17) and 9.4 % (n=5) showing intermediate and resistant profiles respectively. Likewise, GEN showed 81.1 % (n=43) effectiveness against *E. coli* while 1.9 % (n=1) was resistant with 17.0 % (n=9) showing intermediate susceptibility pattern.

Table 4: Percentage (%) of Antibiotics Sensitivity of *Escherichia coli* isolated from the Snails

Antibiotics	Choba (n=20)			Rumuokoro (n= 22)			Oyigbo (n=11)			Overall sensitivity report across all markets (N=53)		
	R	I	S	R	I	S	R	I	S	R	I	S
CRX	20(100)	0(0)	0(0)	22(100)	0(0)	(0)	11(0)	(0)	0(0)	53(100)	0(0)	0(0)
AUG	20(100)	0(0)	0(0)	22(0)	0(0)	0(0)	11(0)	0(0)	0(0)	53(100)	0(0)	0(0)
NIT	3(15)	8(40)	9(45)	2(9.09)	6(27.3)	14(63.6)	0(0)	3(27.3)	8(72.7)	5(9.4)	17(32.1)	31(58.5)
CPR	0(0)	0(0)	20(100)	0(0)	0(0)	22(100)	0(0)	0(0)	11(100)	0(0)	0(0)	53(100)
CAZ	20(100)	0(0)	0(0)	22(100)	0(0)	0(0)	11(100)	0(0)	0(0)	53(100)	0(0)	0(0)
GEN	1(5)	6(30)	13(65)	0(0)	3(13.6)	19(86.4)	0(0)	0(0)	11(100)	1(1.9)	9(17.0)	43(81.1)
CXM	20(100)	0(0)	0(0)	22(100)	0(0)	0(0)	11(100)	0(0)	0(0)	53(100)	0(0)	0(0)
OFL	0(0)	0(0)	20(100)	0(0)	0(0)	22(100)	0(0)	0(0)	11(100)	0(0)	0(0)	53(100)

Key: AUG; Augmentin, NIT; Nitrofurantoin, CPR; Ciprofloxacin, CAZ; Ceftazidime; GEN.; Gentamicin, CXM.; Cefixime, OFL.; Ofloxacin, CTR.; Ceftriaxone, ERY; Erythromycin, CXC.; Cloxacillin. CRX; Cefuroxime; (0-13mm- Resistant (R), 14-16mm - Intermediate (I), 17mm> Sensitive)

Prevalence of Virulent Genes among E. coli Isolated from Raw and Ready-to-eat Snails

Of the three (3) virulence genes investigated (*eae*, *ast* and *aggR* genes) only one isolate was positive for the virulence genes *aggR* representing 1.96% (1 of 51).

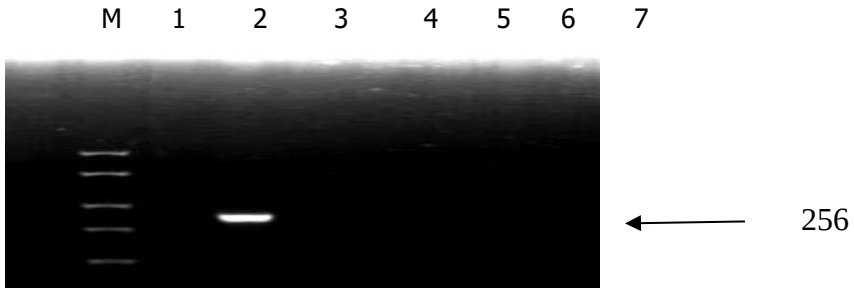

Plate 1.1: Product obtained when total genomic DNA of *E. coli* was subjected to PCR using aggR virulence gene. M is 100kb DNA ladder. Lane 1, 3, 4, 5 and 6 are negative samples while lane 2 is positive.

Discussion

Snail, as meat source that is widely consumed in South-south Nigeria, should meet acceptable standard quality in terms of absence of pathogen, to forestall the outbreak of foodborne disease. In this study, raw and ready-to-eat land snail (*Archachatina marginata*) samples vended in selected markets within Port Harcourt were analyzed for their proximate composition and the presence of *Escherichia coli*. The results revealed some differences in nutritional composition between the raw and ready-to-eat snail samples which might arise from processing.

The high moisture content (78.32%) recorded in this study for raw snail is comparable with 66.27%, 67.70%, and 76.67%, previously reported (Imeybore, 1990; Salyers et. al., 2004; Abd El-Baky et. al., 2020). The 2.5% lipid content recorded in this study for raw snails is also comparable with 1.38% reported by Fagbuaro (2006) but however lower than the 5.67%, 6.58% and 8.86% reported by Edidiong et. al. (2016) and Engmann et. al. (2013) and Raima et. al. (2019) in their respective studies.

There were clear distinctions between 10.01% protein content of raw snail reported in this study and data reported by Edidiong et. al. (2006) and Engmann et. al. (2013) who reported 60.56% and 82.96% respectively in their separate studies. These differences may be attributed to the kind of feeds made available during rearing (Emelue et. al., 2013). The protein content reported in this study was lower than the 20.34 % and 26.34 % reported by Raima et. al. (2019) and Fagbuaro (2006) in their findings.

The 3.29 % Carbohydrate content recorded in this study was higher than the 2.63% but not comparable with 0.37% reported in separate works by Raima et. al. (2019) and Fagbuaro et. al. (2006) respectively. It was however observed that the 11.6% carbohydrate content recorded in the present study for ready-to-eat samples was higher than the 3.26% reported by Engmann et. al. (2013) in ready-to-eat sample. This disparity may be due to the method of processing adopted as it has been reported that the kind of processing done on snail meat may influence the value of nutrient available (Emelue et. al., 2013; Akpomie et. al., 2019).

Escherichia coli count showed varying loads for the different samples sourced from the different markets studied. Raw snails sourced from Choba market were all positive for *E. coli* with counts ranging from $1.8x10^4$ to $8.2x10^5$ CFU/g. It was however observed that all the ready-to-eat samples purchased from Choba and environs were free of *E. coli* contamination indicating that proper processing as well as hygienic handling were maintained during processing.

Similarly, all raw snail samples from Rumuokoro showed one hundred percent (100 %) presence of *E. coli* with counts ranging from $4.2x10^4$ to $6.9x10^6$CFU/g. Ready-to-eat snail samples from same location showed that two of the ten samples studied were contaminated with *E. coli* and had counts of $3.9x10^3$ and $3.5x10^3$CFU/g respectively. Similar to the contamination of raw snails with *E. coli* observed in Choba and Rumuokoro markets, raw snail samples obtained from Oyigbo had 100% occurrence of *E. coli* while the ready-to-eat samples had just one positive representing a 20% occurrence of *E. coli*.

The prevalence of *E. coli* in ready-to-eat snail samples recorded in this study was 12% (n=3).

However, all raw samples from the various markets studied were positive for *E. coli*. The 12.0 % (n=3) prevalence of *E. coli* recorded in this study is however lower than the 17.7 % (n=8) reported by Adagbada et. al. (Peterson & Bonomo, 2005) in ready-to-eat snails sold across Cross Rivers and Akwa Ibom States, South-South Nigeria.

The total resistance of both *E. coli* to Ciprofloxacin and ofloxacin recorded in this study agrees with the 100% resistance reported by Daminabo et. al. (2020) in Port Harcourt. The 100 % (55) resistance of *E. coli* to augmentin reported in this study agrees with the report of Adebayo-Tayo et. al. (2012) who reported a 100% (3) resistance in *E. coli* to augmentin. However, their report of 100% (3) *E. coli* resistance to gentamycin does not agree with the 1.9% (1) recorded in the present study. Differences in sample size and antibiotic concentration in our study could be reasons for disparity. The inability to control the emergence of multi-drug (MDR), extensive drug (XDR) and pan drug resistance will increase the mortality rates to 10 million people by 2050 (Abd El-Baky et. al., 2020).

Escherichia coli isolated in this study showed no band for *eae* and *ast* genes. However, only one isolate representing 1.96 % (1) showed band for *aggR* genes. Deji-Agboola et. al. (2019) has however reported the presence of *eae*, hly, *stx*1, *stx*2, *rfb*E and *fli*CH gene in *E. coli* isolated from raw and ready-to-eat meat samples in Nigeria.

Conclusion

Raw snail samples were highly contaminated with *Escherichia coli.* The *E. coli* isolated were resistant to at least one of the antibiotics tested. The *aggR* gene was the only virulent gene detected in one isolate. The presence of *E. coli* in processed snails' meat obtained in this study strongly suggests the urgent need to improve the quality control and assurance systems.

Acknowledgement

The authors are grateful to the technical personnel of Emadavistic Laboratory, Osaks House, East-West Road Port Harcourt and Prof. B. O. Agaviezor.

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