Food Science and Technology 7(3): 23-30, 2019
DOI: 10.13189/fst.2019.070301 http://www.hrpub.org

Occurrence and Enumeration of Multiple Bacterial Pathogens in Edible Snails from South East Nigeria

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Abstract Edible snails are usually obtained from the forest and in high demand among consumers. Data on the level of contamination of edible snails with bacterial pathogens are needed for making legislations that will improve food safety and protect public health. This study aimed to determine the occurrence and distribution of counts of selected bacterial pathogens in *Achatina achatina* from major markets within South East Nigeria. A total of 300 samples of *A. achatina* were examined for occurrence and counts of *Citrobacter, Shigella, Escherichia coli, Staphylococcus, Aeromonas* and *Bacillus cereus* using enrichment broth, differential and selective media. Snail samples from Ogbete market had the highest mean aerobic plate count (9.32 ± 0.308 Log CFU/g), while Abakaliki market samples had highest mean count of coliforms (7.63 ± 0.389 Log CFU/g). Among pathogens, highest counts were observed for *Citrobacter* and *E. coli* which ranged from 6.0 to 8.0 Log CFU/g in 300 (100%) and 180 (60%) samples respectively. Significant differences were observed among the locations ($p < 0.01$). Our findings highlight the need for formulation and implementation of strategies for the reduction of bacterial pathogens in edible snails along the value chain.

Keywords *Achatina achatina, Aeromonas, Bacillus cereus, Citrobacter, Escherichia coli, Staphylococcus*

1. Introduction

Foodborne diseases cause significant burden of disability and mortality in most countries. Diarrhoeal diseases are the most common illnesses resulting from the consumption of contaminated foods, causing 550 million people to fall ill and 230,000 deaths annually (WHO, 2017). Currently, Nigeria's diarrhoea prevalence is 18.8% according to the joint report from the federal ministries of Agriculture, Environment and Health (FMAEH, 2017).

Food poisoning and diarrhoea caused by foods contaminated by *Citrobacter* have been reported (Doulgeraki et al., 2011). Currently, the incidence of shigellosis worldwide is highest among children less than five years of age (Taneja and Mewara, 2016). *E. coli* is one of the major foodborne pathogens of foods of animal origin with wide variability of virulence (Kobayashi et al., 2002; Johnson et al., 2005). *Aeromonas* have been associated with several food-borne outbreaks and are progressively being isolated from patients with traveler's diarrhoea (Von Graevenitz, 2007). The true burden of illnesses caused by *B. cereus* is unknown probably because they commonly occur as sporadic cases, rather than in major outbreaks (Logan et al., 2011).

All food groups contribute to the burden of foodborne diseases, and foods of animal origin cause the highest burden (Havelaar, 2016). Carriage and shedding of zoonotic pathogens contaminate the environment and eventually enters the food chain during processing and post processing procedures (Coker et al., 2000). However, most people are inadvertently exposed to microbial hazards from several other sources (Okafor et al., 2017; Amini et al., 2012), which can cause diseases that go unreported. The proportion of the national burden of disease linked to the environment in Nigeria is 29% (WHO, 2009).

Snails are consumed by the people of both rural and urban communities (Efuntoye et al., 2011). The most common species in West Africa is *Achatina achatina* (Hodasi, 1984). Consumption level of snail meat among people of Bori (a southern city in Nigeria) is as high as 70% (Nodu et al., 2003). The global snail market has recorded a turnover of 10 billion Euros per year with consumption of about 400,000 tons of snails, of which only 50,000 tons were produced in snail farms (Toader, 2012).

However, mild gastroenteritis has been reported to be common among people that consume snails regularly (Serrano et al., 2004). Bacterial pathogens have been detected from varieties of snails in previous studies (Adegoke et al., 2010; Omenewa et al., 2011, Adagbada et al., 2011; Ebenso et al., 2012; Nyoagbe et al., 2016). However, there is limited data on the comparative

distribution of viable counts of multiple bacterial pathogens among snails displayed for sale in markets in more than two states in Nigeria. Such data is important for making legislations that will improve food safety.

The objective of this study was to determine the occurrence and distribution of the viable counts of *Citrobacter*, *Shigella*, *E. coli*, *Staphylococcus*, *Aeromonas* and *Bacillus cereus* in *A. achatina* from major markets in three states within South east geopolitical zone of Nigeria.

2. Materials and Methods

2.1. Collection of Snail Samples

A total of 300 samples of live edible snails (*Achatina achatina*) were randomly collected from markets in south east, Nigeria and analysed: comprising of hundred samples each from three states in south east geopolitical zone of Nigeria, namely Anambra, Ebonyi and Enugu. Central markets serving as the largest platforms for sale of live edible snails in these states were selected for this study. They were: Ogbete main market at Enugu State, Abakaliki meat market at Ebonyi State and Nkwo Igboukwu market at Anambra State. Samples were collected from July 2016–December 2016 and April 2017–June 2017.

Edible Snails (*A. achatina*) were identified according to their shape, size, markings, colour, spire angle, sculpture and aperture form (Igbinosa et al., 2016; Raut and Barker, 2002). Edible snails displayed on the tables for sale were aseptically collected in plastic containers sterilized with 70% alcohol and dried with commercially available sterile paper towel. Samples were quickly transported to the laboratory for analysis.

2.2. Sample Preparation

The shells of the snails were surface sterilized with 70% ethanol before being aseptically shucked with a sterile iron rod to extract the meat. The lab blender was sterilized with 70% ethanol. The sample (50 g) was homogenized in 450 ml of Ringers solution (Oxoid) using the lab blender for 2 mins at medium speed. The homogenate was used for serial dilution (1:10). Aliquot (1 ml) of appropriate dilution factor was used for determination of bacterial counts

2.3. Determination of Total Aerobic Plate Count

Plate count agar (Oxoid) was prepared according to manufacturer's instructions and maintained at 45^0C. Aliquot (1 ml) of appropriate dilution factor (10^{-6}-10^{-8}) of the homogenate was pipetted into sterile petri dish and the molten agar media was poured into the petri dish. The plate was swirled to mix the homogenate with the agar media. It was done in triplicates for each sample. Plates were incubated aerobically at 37^0C for 24 hours after which colonies were counted and recorded.

2.4. Determination of Coliform Count

MacConkey agar (Titan) was prepared according to manufacturer's instructions. Aliquot (0.1 ml) of appropriate dilution factor was plated out on the agar medium. It was done in triplicates for each sample. Plates were incubated aerobically at 37^0C for 24 hours after which pink colonies were counted and recorded.

2.5. Determination of Viable Counts of Bacterial Pathogens

All agar media namely: Salmonella-Shigella agar (Biotech), Eosin Methylene Blue agar (Oxoid), Mannitol Salt agar (Oxoid), Thiosulfate Citrate Bile salt Sucrose agar (Titan) and Brain Heart Infusion agar (Titan) were prepared according to manufacturer's instructions.

Aliquot (0.1 ml) of appropriate dilution factors was directly plated out on appropriate agar media, except Brain Heart Infusion agar, specific for each pathogen and incubated aerobically at 37^0C for 24 hours. It was done in triplicates for each sample. Typical colonies were counted and recorded after 24 hours. However, appropriate dilution factors were heated in a water bath at 80^0C for 10 mins before being plated on Brain Heart Infusion agar. It was done in triplicates for each sample. Plates were incubated aerobically at 37^0C for 24 hours after which typical colonies of *Bacillus cereus* were counted and recorded.

2.6. Isolation and Identification of Selected Bacterial Pathogens

The procedure for identification of the six genera of bacterial pathogens was based on the United Kingdom Standards for Microbiology Investigations as published by Public Health England (2014 and 2015) and is discussed in the following sections.

2.7. Isolation and Identification of *Citrobacter*

The procedure used was tailored towards isolation of *Salmonella*. Briefly, aliquot (5 ml) of the homogenate was enriched in Selenite fluid (Tulip) (45 ml) for 24 hours, after which a loopful was streaked on Salmonella-Shigella agar (Biotech) and aerobically incubated at 37^0C for 24 hours. Presumptive colonies (white colonies with black centres) were subcultured in Tryptose Soya agar (Oxoid) and subjected to further tests such as Gram staining, catalase test, motility test, oxidase test, indole test, urease test and triple sugar iron test. Representative isolates were forwarded to International Institute of Tropical Agriculture, Ibadan for confirmation of identity using 16S rRNA gene sequencing technique.

2.8. Isolation and Identification of *Shigella*

Presumptive colonies (white colonies without black centres) on Salmonella-shigella agar (Biotech) were subcultured in Tryptose Soya agar (Oxoid) and subjected to further tests such as Gram staining, catalase test, motility test, oxidase test, urease test and glucose fermentation test.

2.9. Isolation and Identification of *Escherichia coli*

Aliquot (5 ml) of the homogenate was enriched in lactose bile broth (20 ml) for 18 hours, after which a loopful was streaked on Eosin Methylene Blue (EMB) agar (Oxoid) and aerobically incubated at 37^0C for 24 hours. Presumptive colonies (blue-black colonies with green metallic sheen and dark centres) on EMB agar were streaked on sorbitol MacConkey agar (Titan) and subcultured in Tryptose Soya agar (Oxoid) and subjected to further tests such as Gram staining, catalase test, indole test, urease test and citrate test, haemolysis test.

2.10. Isolation and Identification of *Staphylococcus*

Aliquot (5 ml) of the homogenate was enriched in Nutrient broth (Oxoid) containing 3% NaCl (20 ml) for 24 hours, after which a loopful was streaked on Mannitol Salt agar (Oxoid) and aerobically incubated at 37^0C for 24 hours. Presumptive colonies (yellow colonies) were subcultured in Tryptose Soya agar (Oxoid) and subjected to further tests such as Gram staining, catalase test, coagulase test and haemolysis test.

2.11. Isolation and Identification of *Aeromonas*

Briefly, aliquot (5 ml) of the homogenate was enriched in Nutrient broth (Oxoid) containing 3% NaCl (20 ml) for 24 hours, after which a loopful was streaked on TCBS agar and aerobically incubated at 37^0C for 24 hours. Presumptive colonies (yellow colonies) on Thiosulfate Citrate Bile salt Sucrose agar (TCBS) agar (Titan) were subcultured in Tryptose Soya agar (Oxoid) and subjected to further tests such as Gram staining, oxidase test, motility test, haemolysis test, lecithinase test and gelatinase test.

2.12. Isolation and Identification of *Bacillus cereus*

Aliquot (5 ml) of the homogenate was heated in a water bath at 80^0C for 10 mins and enriched in Brain Heart Infusion broth (Titan) (20 ml) for 24 hours, after which a loopful was streaked on agar and aerobically incubated at 37^0C for 24 hours. Presumptive colonies (raised grey colonies) on Brain Heart Infusion agar (Titan) were subcultured in Tryptose Soya agar and subjected to further tests such as Gram staining, spore staining, motility test, haemolysis test, lecithinase test and gelatinase test.

2.13. Data Analysis

Descriptive statistics such as means and frequencies were used to present some of the findings. All data on plate counts were converted to logarithmic value. Analysis of variance (ANOVA) was performed using statistical software available in *Vassarstat* website.

3. Results

The Mean bacterial loads in 300 snails analysed in this study are presented in Table 1. The mean aerobic plate count of samples ranged from 8.43 - 9.61 Log CFU/g. Samples from Ogbete market had the highest mean total aerobic plate count (9.32 ± 0.308 Log CFU/g) while the lowest mean count was found in Igboukwu samples (8.74 ± 0.312 Log CFU/g). There were significant differences between total aerobic plate counts of all groups of samples analysed ($p < 0.01$). Samples from Abakaliki market had the highest mean count of coliforms (7.63 ± 0.389 Log CFU/g) while Igboukwu samples (7.41 ± 0.191 Log CFU/g) had the least counts and there were no significant differences between Igboukwu and Ogbete samples. The highest mean *Citrobacter* counts was found in Abakaliki samples (7.24 ± 0.210 Log CFU/g) and there were no significant differences between Igboukwu and Ogbete samples ($p < 0.01$). Abakaliki samples were found to contain the highest mean *Shigella* counts (4.61 ± 0.354 Log CFU/g). There were significant differences between *Shigella* counts of all groups of samples analysed ($p < 0.01$). Igboukwu samples contained the highest mean counts of *E. coli* (7.14 ± 0.170 Log CFU/g) and there were significant differences between all groups of samples analysed ($p < 0.01$). *Staphylococcus* was not detected in Igboukwu samples. The highest mean count of *Aeromonas* was found in Ogbete samples (4.80 ± 0.473 Log CFU/g). Abakaliki samples had the highest mean counts of *Bacillus cereus* (4.50 ± 0.136 Log CFU/g).

Samples were found to contain different levels of bacterial loads (Table 2). All samples had total aerobic plate counts $>10^8$ CFU/g. Most samples (86.7%) had coliform counts ranging from $>10^6 – 10^8$ CFU/g. While *Citrobacter* counts ranged from $>10^6 – 10^8$ CFU/g in all samples, *Shigella* counts were $<10^4$ CFU/g in 35% of the samples. *E. coli* counts were $>10^6 – 10^8$ CFU/g in 60% of the samples. The staphylococci counts were $>10^4 – 10^6$ CFU/g in 26.7% of the samples. While *Aeromonas* counts were $<10^4$ CFU/g in 43.3%, *Bacillus cereus* counts were $<10^4$ CFU/g in 60% of the samples.

Table 1. Mean bacterial loads in snails for sale in three markets in South East, Nigeria

Bacteria	Igboukwu (Log CFU/g±SD)	Abakaliki (Log CFU/g±SD)	Ogbete (Log CFU/g±SD)
*APC	8.74±0.298[a]	8.88±0.312[b]	9.32±0.294[c]
Coliforms	7.41±0.183[a]	7.64±0.371[b]	7.50±0.342[a]
Citrobacter	6.38±0.086[a]	7.24±0.153[b]	6.37±0.210[a]
Shigella	4.44±0.221[a]	4.60±0.319[b]	4.08±0.549[c]
E. coli	7.15±0.163[a]	6.94±0.176[b]	5.65±0.228[c]
Staphylococcus	-	4.75±0.150[a]	4.66±0.589[a]
Aeromonas	3.11±0.052[a]	3.42±0.191[b]	4.80±0.451[c]
Bacillus cereus	3.26±0.112[a]	4.49±0.132[b]	3.48±0.129[c]

*APC: Aerobic Plate Count, SD: Standard Deviation.

[a-c]Means in the same row with different superscripts are different ($P < 0.01$).

Table 2. Distribution of levels of bacterial loads in 300 snails for sale in three markets in South East, Nigeria

Bacteria	$<10^4$ CFU/g [b]N(%)	$>10^4 – 10^6$ CFU/g N(%)	$>10^6 – 10^8$ CFU/g N(%)	$>10^8$ CFU/g N(%)
[a]APC	-	-	-	300(100)
Coliforms	-	-	260(86.7)	40(13.3)
Citrobacter	-	-	300(100)	-
Shigella	105(35)	75(25)	-	-
E. coli	-	90(30)	180(60)	-
Staphylococcus	30(10)	80(26.7)	-	-
Aeromonas	130(43.3)	100(33.3)	-	-
Bacillus cereus	180(60)	60(20)	-	-

[a]APC: Aerobic Plate Count.

[b]N(%): Number of samples contaminated (Percentage of samples contaminated with bacterial load).

Table 3. Prevalence of bacterial pathogens in snails for sale in three markets in South East, Nigeria

Bacteria	Igboukwu (%)	Abakaliki (%)	Ogbete (%)	Total [a]N(%)
Citrobacter	100	100	100	300(100)
Shigella	40	60	80	180(60)
E. coli	90	80	100	270(90)
Staphylococcus	-	30	80	110(36.7)
Aeromonas	70	70	90	230(76.6)
Bacillus cereus	70	80	90	240(80)

[a]N(%): Number of samples contaminated (Percentage of samples contaminated with pathogen).

All 300 samples of market snails analysed in this study were found to be contaminated with pathogens irrespective of the source of the samples (Table 3). *Citrobacter* was detected in all samples across the three sources. *Shigella* was recovered from 60% of all samples analysed, with the highest prevalence in Ogbete samples (80%). *E. coli*, *Staphylococcus*, *Aeromonas*, and *B. cereus* were recovered from 90%, 36.7%, 76.6% and 80% of all samples respectively.

4. Discussion

A. achatina was the focus of this study because of its conspicuous presence in the southern part of Nigeria. It is consumed by the people of both rural and urban communities (Okafor, 1989; Efuntoye et al., 2011). Land snails aestivate from December of a year to March of the next year, and re-surface during the rainy season (Fagbuaro et al., 2006) which explains why this study was conducted during the rainy season.

The findings of this study demonstrate that most snails sold in markets in Anambra, Ebonyi and Enugu states contain various levels of high loads of bacterial indicators and pathogens. The mean aerobic plate count of samples analysed in this study ranged from 8.43 - 9.61 Log CFU/g. These data appear close to the findings of other related studies: Adegoke et al. (2010) reported total aerobic bacterial count in market snails at Akwa Ibom state was 8.0 Log CFU/g. In Ghana, Nyoagbe et al. (2016) reported

that total viable count ranged from 6.61 to 8.29 Log CFU/g. However, Temelli et al. (2006) found the average total aerobic bacterial count in live snails in Turkey to be 6.85 Log CFU/g. Also, mean aerobic counts varied significantly ($p < 0.01$) between the three states from which samples were collected, probably because of the difference in the nature of soil and debris present in the natural habitats of these snails across these locations.

Aerobic plate count is generally used as a means of assessing the overall microbial quality of raw ingredients (Siragusa et al., 1998). According to ICMSF (1986), the acceptable upper limit of total aerobic bacterial load for seafoods is 5.0 Log CFU/g and this limit has been cited in most research articles till date. It is important to note that all snail samples analysed in this study had total aerobic plate counts $>10^8$ CFU/g. This implies that 100% of market snails analysed pose microbiological risk to handlers and consumers. However, the use of the aerobic plate count as an indicator for the presence of specific pathogens is generally not satisfactory (Miskimin et al., 1976; Siragusa et al., 1998).

Coliform counts are used for assessing the amount of contamination on meat arising from gut contents and are the most frequently studied indicators (Wu et al., 2011). The acceptable upper limit of total coliform is 2.0 Log CFU/g (ICMSF, 1986). In this study, the coliform counts were >2.0 Log CFU/g in all samples analysed. Similar coliform count in snails has been previously reported (Adegoke et al., 2010; Nyoagbe et al., 2016), even though the prevalence of levels of concentrations of coliforms in snails has not been reported in any study. It is appropriate to note that snails discharge their faeces within their habitat (Ibom et al., 2012) and may explain the high loads of coliforms observed in this study.

Citrobacter was detected in all samples across the three sources. This observation is supported by another study in India on the bacterial diversity of the gastrointestinal tract of *A. fulica* using culture-independent and culture-dependent methods. The study also concluded that an apparent feature of bacterial communities in snails' gastrointestinal tract was the abundance of members of the genus *Citrobacter* (Pawar et al., 2012). The highest mean *Citrobacter* count was found in Abakaliki samples (7.24 ± 0.210 Log CFU/g). *Citrobacter* is classically considered a resident commensal of the intestinal tracts of both humans and animals (Guerrant et al., 1976). It is also prevalent in soil and water through contamination from the waste materials of animals. A study concluded that healthy pet turtles are a potential carrier of *C. freundii* (Sabrina-Hossain et al., 2017). Therefore, animals are the probable source of *Citrobacter* around swampy environments where most snails are collected.

Mean *Shigella* counts exceeded 4.00 Log CFU/g in all samples examined. Most studies on snails have reported the presence of *Shigella* without indicating its level of concentration (Adagbada et al., 2011). Several aquatic bodies have been found to contain *Shigella* and aquatic foods may play a role in its transmission if such foods are harvested from sewage-contaminated water (Iwamoto et al., 2010). The number of *Shigella* cells required to initiate infection ranges from 10^1 - 10^4 cells/person (Dupont et al., 1989; Heymann, 2004). Since 60% of snails in our study exceeded 10 CFU/g, it implies such percentage of snails represent health threat to handlers and consumers, especially children less than five years of age (Taneja and Mewara, 2016).

For *E. coli*, the highest mean counts were found in Igboukwu samples (7.14 ± 0.170 Log CFU/g), while Ogbete samples had the lowest mean counts (5.65 ± 0.239 Log CFU/g). Sixty percent of the snails in this study had *E. coli* counts > 6.0 Log CFU/g which is within the range of counts prominent for resulting in diarrhoeal diseases 6.0 – 9.0 Log CFU/g (Kornacki and Marth, 1982).

The mean staphylococcal count in samples analysed in this study ranged from 4.66 – 4.74 Log CFU/g. The only study that quantified the level of *Staphylococcus* in snails reported a range between 2.66 and 7.68 Log CFU/g (Nyoagbe et al., 2016). Diagnosis of staphylococcal food poisoning is generally confirmed by the recovery of at least 5.0 Log CFU/g from food (Halpin-Dohnalek and Marth, 1989; Hennekinne et al., 2012). It is suggested that since *Staphylococcus* is also present in intestinal tract, meat may contain *Staphylococcus* resulting from contamination with intestinal content during evisceration (Bhalla et al., 2007). It is not clear why *Staphylococcus* was not detected in Igboukwu samples, but may have been present at very low counts.

The mean count of *Aeromonas* was found to range from 3.10 - 4.80 Log CFU/g. The infectious dose of *Aeromonas* species in foods is not known (Isonhood and Drake, 2012). In another study, mesophilic aeromonads were isolated from 26% of vegetable samples, 70% of meat and poultry samples, and from 72% of fish and shrimps. Numbers of motile aeromonads present in these samples varied from < 2.0 to > 5.0 Log CFU/g (Neyts et al., 2000). While in our present study, 76.6% of the snail samples contained *Aeromonas*. This is important because snails feed on assortment of plant and animal species including algae (Okafor, 1989). Formulated feeds for snails are not available in the market. Therefore, it has become common practice for snail rearers to use vegetables, plant leaves and kitchen wastes to feed snails (Chah and Inegbedion, 2013). *Aeromonas* species are widely distributed in the aquatic environment (Palumbo, 1996, Neyts et al., 2000) and their prevalence in various water and food sources represents a significant public health threat (Wu et al., 2007). Edible snails sold in the markets are usually obtained from the forest and are in high demand among consumers (Nyoagbe et al., 2016). Also, they are usually purchased alive in the market by consumers and brought into homes where they are handled and prepared in the domestic kitchens.

The results of this study indicate that *Bacillus* counts for

Abakaliki samples had the highest mean count of *Bacillus* (4.50 ± 0.136 Log CFU/g) followed by Ogbete samples (3.48 ± 0.135 Log CFU/g) and Igboukwu samples (3.25 ± 0.130 Log CFU/g). Nyoagbe et al. (2016) reported similar levels of *Bacillus* in snails ranging from 1.53 to 4.90 Log CFU/g. It is often noted that *Bacillus cereus* levels > 3.0 Log CFU/g always result in illness (Harmon et al., 1992).

5. Conclusions

Snails displayed for sale in markets at Anambra, Ebonyi and Enugu states contain high loads of bacterial indicators and pathogens at high prevalence rate. Among the pathogens studied, highest counts were observed for *Citrobacter* and *E. coli* in samples. Edible snails may play a role in the transmission of foodborne bacterial pathogens in the food chain. Sources of edible snails should be monitored and protected from routes of bacterial contamination. There is need to collaborate with environmental professionals. Our findings highlight the need for formulation and implementation of strategies for the reduction of bacterial pathogens in edible snails along the value chain.

Conflict of Interest

The authors declare there is no conflict of interest.

Author's Contribution

Arthur C. Okafor and Frank C. Ogbo planned and conducted the research work, analyzed and interpreted the data and also drafted the manuscript.

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