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Effect of Different Preparatory Methods on Microbial Load of the Edible Frog *Hoplobatrachus occipitalis* from Aguleri, Anambra State, Nigeria

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Abstract
With the increasing use of the edible frog Hoplobatrachus occipitalis as a delicacy in many parts of Nigeria, and food poisoning risks associated with contaminating microorganisms, it has become necessary to determine the best method of preparing the frog to eliminate/reduce microbial contamination before consumption. Fresh and smoke dried samples were bought from traders in Aguleri, Anambra East Local Government Area, Anambra State, Eastern Nigeria. Some fresh samples were oven dried in the laboratory. Enumeration, isolation, characterisation and identification of bacterial and fungal isolates were carried out using standard microbiological methods. The total heterotrophic bacterial counts for the fresh, smoke dried and oven dried samples were $2.1x10^5$cfu/g, $1.8x10^5$cfu/g and $2.5 x 10^5$cfu/g respectively, while the total fungal counts were $6.2x10^4$ cfu/g for the oven dried and $2.1x10^4$cfu/g for both the fresh and smoke-dried samples. A total of forty-three (43) bacterial and fungal (including yeast) isolates were isolated from fresh samples of Hoplobatrachus occipitalis. The bacterial isolates include Acinetobacter sp., Bacillus sp., Escherichia coli, Flavobacterium sp, Micrococcus sp., Pseudomonas aerogenosa, Serratia sp., Staphylococcus aureus and S. epidermidis while the fungal isolates obtained include Aspergillus flavus, A. niger, A. tamari, Botrytis sp., Cladosporium sp., Geotrichum sp., Mucor sp., Penicillium chrysogenum, P. italicum, P. oxalicum and Trichoderma sp., Yeast isolates include Cryptomonas neoformis, Saccharomyces cereviaves and Saccharomyces sp. The smoke-dried samples had the least bacterial species while the fresh and smoke-dried ones had the least fungal species. Smoke-drying in hygienic conditions is highly recommended for consumers since they had least bacterial and fungal counts. This may greatly reduce the risk of food poisoning that might pose serious health challenges.

Keywords: *Hoplobatrachus occipitalis*, Microbial load, Edible frog, Aguleri,

Introduction
The importance of frog legs in many countries could be seen by the different ways they are used as food coupled with research on how to get the best from them. Various works have been done on the frog legs to either evaluate the meat colour and pigment level using different methods of slaughtering (1) or to determine the volatile components of the bullfrog legs with results showing resemblances in its unsaturated aliphatic aldehydes to that of chicken which according to researchers gives it a similar taste to chicken (2). Other works done on the edible frog included the determination of the shelf life using boiling and frying methods (3). It was discovered that at the end of 13 days the frog legs still remained fresh irrespective of mode of preparation as the microbial load reduced as soon as boiling or frying occurred when compared to the fresh samples. In Brazil, frog legs were used as nutritional additives in babies' food as microbes present were found to be within the limits of Brazilian law (4). In Nigeria, the frogs are eaten whole unlike most western countries where only the legs are sought after. A survey of edible amphibian species in South western Nigeria showed farmers as being the highest consumers of frogs followed by secondary school teachers and then housewives. They also ate not just *Hoplobatrachus occipitalis* but other species as *Ptychadena pumilio* and *Xenopus muelleri* (5). A rich cross boundary trade involving Cameroun, Chad and Nigeria in edible amphibians was recently discovered. In all the study sites, *Hoplobatrachus occipitalis* was the most used for food (6). Specimens of various sizes were dried, put in sacks and carried to other parts of the country for sale, while others were taken out of the country to neighbouring countries where taxes even had to be paid at the borders.
Harmful microbes were recently isolated from *Hoplobatrachus occipitalis* in Igwuruta, Rivers State that could pose health implications to the consumers. They included *Escherichia coli*, *Salmonella typhi*, *Vibrio cholerae*, and *Shigella sp.* (7). With the associated microbes on the edible frog and its popularity as an alternative delicacy as meat in some parts of Nigeria, it has become necessary to determine best preparatory methods to reduce microbes that could pose health challenges.

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Materials and Methods

Study area

The study was carried out in Aguleri, Anambra East Local Government Area of Anambra State. The people of Aguleri belong to the Igbo ethnic group in Nigeria. They are mainly farmers and fishermen, with some businessmen and women. Both fresh and smoked *Hoplobatrcahus occipitalis* samples were bought from Umuoba-Anam riverbank market in Aguleri very close to River Niger. The individuals selling it were interviewed on the habitat, types of species found, their consumption by humans and the processes of preservation of the edible frogs. The samples were transferred to the University of Benin, Benin City, Edo State in a small plastic bucket containing river water while the dried samples were placed in a polythene bag. They were taken to the laboratory for microbiological studies within 72 hours of collection and analyzed for microbial quality.

Fig. 1: Map showing Aguleri in Anambra East Local Government Area

Fig. 2:Umuoba-Anam riverbank market

Enumeration, isolation characterisation and identification of microorganisms.

Serial dilution of the samples was carried out by vortexing the frog samples in sterile 0.1% peptone water as diluent. Twenty grammes (20.00 g) of the sample was weighed out aseptically into 180.00ml of the diluent using a calibrated balance. The weighed sample was then vortexed to produce a 10^{-1} homogenate from which subsequent dilutions of up to 10^{-7} were made. Bacterial and fungal culturing were carried out using the pour plate technique. The total heterotrophic bacterial count was determined by using 1.0ml of appropriate serially diluted samples in nutrient agar (Oxoid Ltd., Hants, England) with antifungal agent (0.5 ml of griseofulvin solution per plate) incorporated to inhibit fungal growth. The fungal (moulds and yeasts) enumeration was determined using potato dextrose agar (Oxoid Ltd., Hants, England) to which 0.5 ml of an antibacterial agent was incorporated. The antifungal agent was prepared by dissolving 300 mg of griseofulvin in 12.5 ml of sterile distilled water. The antibacterial agents (2.5 g penicillin and 1.0 g streptomycin) were dissolved in 30.0 ml sterilised distilled water. Triplicate plates of appropriate dilutions were made. The culture (Petri-dishes for bacteria and fungi) were incubated at room temperature (28 $\pm$ 2^0C) for 48-72h for colonies to develop. The means of colony counts were used to compute colony forming units per gram (cfu/g) of total heterotrophic bacterial and fungal counts of the frog samples analysed (8).

Results

Total Viable Count

The result of the total heterotrophic bacterial and fungal propagules counts of all the samples obtained from this location are shown in Tables 1 and 2. Generally, it was observed that the total estimated viable heterotrophic bacterial count was higher than those of the total fungal count. The highest mean bacterial count was 2.5 x 10^5cfu/g for the oven dried samples, while the lowest was 1.8 x 10^5cfu/g for the smoke dried ones. The oven dried samples also recorded the highest mean fungal count of 6.2 x 10^4cfu/g while the fresh and smoke dried samples recorded the least (2.1 x 10^4cfu/g).

Plate 1: Fresh *Hoplobatrachus occipitalis* samples

Plate 2: Smoke-dried *Hoplobatrachus occipitalis* samples

Table 1: Total viable heterotrophic bacterial counts (cfu/g) of *Hoplobatrachus occipitalis* treated with different preparatory methods from Aguleri, Anambra State

	Heterotrophic bacterial counts (cfu/g)		
Samples	**Fresh**	**Smoke-dried**	**Oven-dried**
1.	1.8×10^5	1.5×10^5	2.2×10^5
2.	2.2×10^5	1.8×10^5	2.4×10^5
3.	2.4×10^5	2.0×10^5	2.8×10^5
$\bar{X}$	**2.1×10^5**	**1.8×10^5**	**2.5×10^5**

Table 2: Total fungal propagule counts in colony forming units (cfu/g) per gram

	Fungal propagule counts (cfu/g)		
Samples	**Fresh**	**Smoke-dried**	**Oven-dried**
1.	3.0×10^4	3.4×10^4	1.5×10^5
2.	1.8×10^4	1.7×10^4	1.5×10^4
3.	1.4×10^4	1.3×10^4	2.2×10^4
$\bar{X}$	**2.1×10^4**	**2.1×10^4**	**6.2×10^4**

Frequency of Occurrence

The frequency of occurrence of microbial isolates from *Hoplobatrachus occipitalis* sample obtained from Aguleri in Anambra State is shown in Table 3. A total of forty-three (43) microbial isolates identified as bacteria and fungi.

Table 3: Synopsis of microbial isolates of *Hoplobatrachus occipitalis* samples obtained from Aguleri, Anambra East LGA ,Anambra State with their frequency of occurrence

Isolates	**Frequency of occurrence (%)**
Bacterial isolates	
*Acinetobacter*sp.	66.67
Bacillus sp.	33.33
Escherichia coli	66.67
Flavobacterium sp.	33.33
Micrococcus sp.	100.00
Pseudomonas aeruginosa	33.33
Saccharomyces sp.	33.33
Serratia sp.	66.67
Staphylococcus aureus	100.00
S. epidermidis	100.00
Fungal Isolates	
Aspergillus flavus	66.67
A. niger	100.00
A. tamari	33.33

Botrytis sp.	33.33
Cladosporium sp.	33.33
Cryptomonas neoformis	33.33
Geotrochum sp.	33.33
Mucor sp.	66.67
Penicillium chrysogenum	33.33
P. italicum	100.00
P. oxalicum	100.00
Penicillium sp.	33.33
Trichoderma sp.	33.33
Yeast (*Saccharomyces)*	66.67

Micrococcus sp., *Staphylococcus aureus* and *S. epidermidis* have the highest frequency of occurrence for bacteria counts while *Aspergillus niger*, *Penicillium italicum* and *P. oxalicum* have the highest frequency counts for fungi. Most microbial isolates have low frequency of occurrence (33.33%) and includes *Saccharomyces* sp., *Bacillus* sp., *Flavobacterium* sp., *Pseudomonas aerogenosa*, *Trichoderma* sp., *Geotrichum* sp., *Botrytis* sp., *Penicillium chrysogenum*, *Aspergillus tamari*, *Cladosporium* sp., *Cryptomonas neoformis* and *Penicillium* sp. The most frequently isolated bacteria from the frog sample were identified as *Micrococcus* sp., *Staphylococcus aureus* and *S. epidermidis* while the most frequently isolated fungi were identified as *Aspergillus niger* and *Penicillium oxalicum.*

Discussion

The results of this investigation revealed that the quality, quantity and frequency of occurrence of microbial isolates in *Hoplobatrachus occipitalis* samples collected from Aguleri in Anambra State studied were high. Most of the organisms isolated are likely to be pathogenic since the origin is from man either as skin microorganisms or as coliforms, which have specific pathogenic tendencies (ability to cause diseases especially when consumed at high rates) (9). The bacteria isolated from the frog samples include *Acinetobacter* sp., *Bacillus* sp., *Escherichia coli, Flavobacterium* sp., *Micrococcus* sp., *Pseudomonas aeroginosa*, *Staphylococcus aureus*, *S. epidermidis* and *Serratia* sp. Association of these organisms with the frogs is of great significant health importance as their presence suggested a high level of contamination indicating the possible risks of food poisoning to human consumers (10). Faecal coliforms in the frog samples is a natural occurrence and difficult to remove before preparation (3). This coupled with inadequate hygiene, mishandling and improper storage could lead to an increase in the microbial load of the frogs. The presence of coliforms on the frogs could be attributed to faecal contamination, which results in food borne diseases like cholera and dysentery if not properly handled while cooking. The aged, children, as well as immune-compromised patients may be adversely affected with consumption of improperly processed *Hoplobatrachus occipitalis* with great economic losses and loss of man-hours (7). The location of the market by the river bank may have also played a role in its contamination as people use the river as a quick alternative to toilets. The different preparatory methods showed smoke drying as the best method of reducing the bacterial load on the frogs compared to the oven-dried ones, while for the fungi, smoke drying made no difference as both the smoke-dried and fresh samples had the same values. Consumers in the United States, Canada and France however preferred the frog meat fresh even though this was more expensive with a lower shelf life. They may thus have more microbes to contend with. The taste of fresh frog legs were found to be similar to chicken (11). This may be the driving factor in the demand for frogs as the frog trade cut across the country into neighbouring countries (6). In Aguleri market however, people bought the frog meat based first on availability and then preference. The increase in the microbial load of the oven dried samples could be as a result of poor handling during processing. Smoke - dried frogs would however not stay fresh after 15 days despite it being smoked (12). A high level of hygiene should be observed in the preparation of these frogs for food to reduce contamination and possibly food poisoning.

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