Microbiological quality of edible frogs and proximate composition analysis

Abstract

Introduction:The frog (*Pelophylax esculentus*) is an edible frog and its meat is popular in some parts of the world, especially in West African countries. The frog meat has protein nutrient content in the diet. Aim: The aim of this work is to find the microbiological quality and analyze the proximate composite value of Pelophylax esculentus and the objectives are to, isolate and determine the total heterotrophic count of bacteria and fungi associated with the meat, to also analyze and determine their nutritional contents. Result: Results indicate the total heterotrophic bacterial count of $7.0x10^7$cfu/g while total fungal, were $5.9x10^7$cfu/g isolated from the gut; $6.8x10^6$cfu/ml, total bacteria count and total fungal count of $5.2x10^6$cfu/ml was from the skin while $7.3x10^6$cfu/ml and $4.5x10^6$/ml total bacteria and fungal count respectively from the mouth.The various bacteria and fungi isolated from the samples includes; *Vibrio parahaemolyticus., Vibrio cholera. Shigalla.spp., Salmonalla spp., Escherichia coli., Staphylococcus spp., Bacillus spp., Klebsiella spp., Pseudomonas spp.*, and also fungal isolates includes; *Aspergillus spp., Penicillium spp., Candida spp., Cryptococcus spp*. While the Proximate Composition indicates; protein (45.06%), moisture (40.27%), ash (5.70%), carbohydrate (3.00%), fat (2.55%), and fibre (3.42%) respectively. Conclusion: This frog species is a very good source of protein as a meat delicacy compared to some other animal meat. Hence, it can be recommended in the diet of people lacking protein, especially where other animal meat is scarce or expensive. Based on some species of microorganisms discovered during the study that can pose as a threat to human health/life, hence the meat needs proper cooking and handling before consumption.

KEYWORDS: **Microbial quality, edible frog, proximate composition, microbial counts, isolation**

1. INTRODUCTION

The frog (edible frog),belongs to the Kingdom animalia, Phylum chordata, Class amphibian, Order Anura, family Ranidae, Genus *Pelophylax,* Species *P. esculentus*, *P. lessonae* and *P. ridibundus*. Their meat is becoming very popular as a source of protein and other medicinal delicacies in many West African countries, including Nigeria (Douglas and Amuzie, 2017). Frogs have been used in the production of infant food (Rodrogues*et al*., 2014). Frog meat is also a delicacy in parts of Rivers State where they are harvested from the forests and temporary ponds in water it is logged areas. All the *Ptychadena* species are found in Rivers State, which include *P. mascareniensis, P. oxyrhynchus, P. pumilio, P. bibroni, P. schubotzi and P. longirostris*and the African bullfrog, *H. occipitalis*, are consumed in Gokana Local Government Area Rivers State of Ogoni (Biara) ethnic tribe. In parts of Oyo State of Nigeria, similar species are also consumed: the gut is removed and discarded while the rest of the

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animal is cooked, fried or salted and roasted for consumption. The rest of the frog is pinned to sticks and smoked. These are then sold in their local markets for consumption.

The local markets in certain areas of Oyo State, Nigeria, sell similar species of frog, where the gut is discarded and the remaining parts are either cooked, fried, salted, and roasted, while the rest of the frog is smoked and pinned to sticks for consumption or before selling them in the local markets. The meat serves as food as well as a source of income or foreign exchange (Douglas and Amuzie, 2017).

They are reared commercially in countries like Malaysia, Taiwan, Indonesia, Brazil and Mexico for human consumption, while others such as USA, France, Canada, Belgium, Italy and Spain are the major importers of frog meat (Ho *et al*., 2008; Baygar and Ozyur, 2010). In most of these countries, the frog legs (the most fleshed part of the frog) are the main parts consumed, believed to be a delicacy. These organisms are consumed in large amounts in European countries. High consumption rates were reported in Italian and French restaurants and in holiday villages in Turkey. (Baygar and Ozyur, 2010).

AIM: The aim of this work is to find the microbiological quality and analyze the proximate composite value of *Pelophylax esculentus*.

OBJECTIVES: The specific objectives are to:

1. isolate and determine the total heterotrophic count of bacteria and fungi associated with the meat
2. analyze and determine their nutritional contents

2. MATERIALS AND METHODS

Experimental Design

This study was based on a cross-sectional design with animal sample collected at a time, organs needed processed and analysis on microbial and proximate composition carried out.

Sample Collection

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Live adult samples of *Pelophylax esculentus* were collected from slow running, semi-stagnant water from Gokana Local Government Area of Rivers State, Nigeria. The samples (*P.esculentus*) were stored and transported to the laboratory, using sterile containers that contain water. The body samples were washed using sterile distilled water to remove transient organisms and dissected within 24hr of collection using standard scientific and ethical procedures. The guts of the sample were removed and kept in a refrigerator at 4^oC in the laboratory until they were needed. The same samples were collected from the same environment following the method of collection three times.

MEDIA USED AND PREPARATION OF DILUENT

The following selective/differential media were used for the isolation of the sample; Nutrient Agar (NA), Mac-Conkey Agar (MCA), Salmonella- Shigella Agar (SSA), Eosin Methylene Blue Agar (EMBA), Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar and Potato Dextrose Agar (PDA). All media were prepared according to manufacturer's directions for the cultivation and isolation of the isolates i.e.28g of Nutrient Agar, 52g of Mac-Conkey Agar,60g of Salmonella-Shigella Agar,36g of Eosin methylene blue Agar,86g of Thiosulfate-Citrate-Bile Salts-Sucrose Agar, and 39g of Potato Dextrose Agar were all dissolved in 1000ml of distilled water (Allen, 2005)

The diluents were equally prepared by adding 0.85g of sodium chloride ($NaCl_2$) to 100ml of sterile distilled water for the preparation of the normal saline.The already prepared media and the diluents (normal saline) were sterilized in an autoclave at 121^oC for 15minutes before they were used. Also, the glass wares were sterilized in a hot oven at 160^oC for 1 hour (Allen, 2005).

ORGAN ISOLATION PROCEDURES

(i) Isolation of the Skin Organisms

With sterile distilled water, the body of each frog was washed thoroughly; this was done to remove transient organisms. After washing, a sterile swab stick was used to swab the body of the frog. The dorsal and the ventral surfaces were all swabbed severely. 2ml of sterile normal saline was added to each of the swab sticks (Douglas and Amuzie, 2017).The swab stick was then shaken vigorously to dislodge the skin microorganisms. After shaking, a further 10-fold serial dilution method by Harrigan and McCance (1990) was carried out using a sterile pipette to transfer 1ml of the initial dilution to 9.0 ml of an appropriate diluent (contains

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normal saline). Finally 0.1ml of an appropriate diluent(aliquot) was spread on the surface of these various agar;(Nutrient agar, Mac-Conkey agar (MCA), Salmonella- Shigella agar (SSA), Eosin methylene blue agar (EMBA), Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar and Potato dextrose agar (PDA)using sterile hockey stick(glass spreader).Plates were incubated at 37°C for 24 to 48 hours. After incubation, counts were performed for discrete colonies that developed on the plate for those dilutions, which showed counts between 30-300 colonies. (Anonymonus,1994).

(ii) Isolation of Mouth Organisms

The mouth of the sample was opened gradually using a sterile forceps and swabbed the mouth with a sterile swab stick. A sterile pipette was used to collect 2ml of sterile normal saline and was added to each of the swab sticks. The swab stick was then shaken vigorously to dislodge the skin microorganisms (Culp,*et al*. 2007). After shaking, 10-fold serial dilutions were carried out using a sterile pipette to transfer 1ml of the initial dilution to 9.0 ml of an appropriate diluent (contains normal saline). Finally 0.1ml of an appropriate diluent (aliquot) was spread on the surface of these various agar;(Nutrient agar, Mac-Conkey agar (MCA), Salmonella- shigella agar (SSA), Eosin methylene blue agar (EMBA), Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar and Potato dextrose agar (PDA) using sterile hockey stick(glass spreader).Plates were incubated at 37°C for 24 to 48 hours. After incubation, counts were performed for discrete colonies that developed on the plate for those dilutions, which showed counts between 30-300 colonies. (Anonymonus,1994).

(iii) Isolation of Gut Organisms

The frog was dissected to get the intestinal content. Then, the internal content was crushed and homogenized, 1g weighed and added to 9ml of normal saline, shaking vigorously to mix and serially diluted to 10^{-8}. A volume of 0.1ml (aliquot) was dispensed from 10^{-7} to 10^{-8} dilution tubes,was spread on the surface of the dried plates of difference Agar; (Nutrient agar, Mac-Conkey agar, Salmonella- Shigella agar, Eosin methylene blue agar, Thiosulfate-Citrate-Bile Salts-Sucrose agar, and Potato dextrose agar). Fungal counts were done using potato dextrose agar (supplemented with 0.5g/l chloramphenicol to inactivate bacteria growth on the agar), while the other media were used for the isolation of coliforms and other enteric bacteria. (Ogbalu and Douglas, 2015, 2016).

(iv) Isolation of Pure Isolates

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The representative colonies that developed on the respective agar plates were counted, picked and inoculated onto a freshly prepared plate (Nutrient Agar), severely until pure isolates were obtained. Pure isolates were stored on nutrient agar slants and refrigerated at 4^oC until they were required for further test.

BIOCHEMICAL CHARACTERIZATION OF BACTERIA AND FUNGI

The colonies that developed on the respective agar plates were counted and subcultured on the respective freshly prepared plates until pure isolates were gotten. Pure isolates were stored on nutrient agar slants and refrigerated at 4^oC until required for further use. Further identification was done based on the cultural, morphological, biochemical and gram reactions according to Cowan and Steel (1966) and also Bergey's Manual of Determinative Bacteriology (Holt,*et al*., 1994). While fungi were identified based on their morphological and microscopic characteristics (Larone, 1995; Barnett and Hunter, 1972).

PROXIMATE COMPOSITIONANALYSIS

(i) Determination of Moisture

The crucibles were cleaned, dried using the air oven for 10 minutes.After drying, the samples were mixed thoroughly and weighed 5g into the crucibles and placed in the oven at 103^oC overnight(24hours).The crucibles were then removed and reweighed after cooling and dried for another 1hour to ensure constant weight.The moisture was calculated using the formula (AOAC, 2000);

$$\% \text{ Moisture} = \frac{\text{Loss of Weight of sample (g)}}{\text{Weight of sample (g)}} \times 100$$

(ii) Determination of Crude Protein

Half a gram of the edible frog was weighed into one quarter size of filter paper, one table of catalyst was added followed by 10ML concentrated sulphuric acid in a digestion flask. The flask was then placed in the heating unit inside the fuming cupboard and heated slowly until the sample boiled. The digestion was done by boiling while agitating the flask until the solution became liquefied and completely clear. The samples were cooled and weighed into a 100ML flask with distilled water. 5ML of boric acid mixed. The indicator solution was transferred into a 100ML clinical flask placed at the end of the condenser of the micro-kjehdhal distillation apparatus so that the adapter was dipped into the liquid.10ML of aliquot was pipetted into a micro kjehhal flask for distillation. 10ML of 45% sodium hydroxide

(i.e.90 in 200ML) was poured carefully down the inclined neck of the solution. The flask was immediately attached to the splash head of the distillation apparatus. Steam was passed through alkaline liquid (i.e. NaOH+ aliquot) slowly until boiled. The liquid was trapped and distilled into 5ML boric acid in the conical flask until 50ML of a distillated from the pipeline was collected to a green colour and then titrated with 0.045N sulphuric acid. The bank was prepared in the same way. Crude protein was calculated using the Kjehhal method(Kjehhal, 1883) as follows:

$$\text{Nitrogen \%} = \frac{\text{Titre-Bank x Normal of acid}}{\text{Weight of sample}} \times \frac{1.4}{1}$$

(iii) Determination of Fat

Two-grams (2g) of the dried samples used for the determination of moisture content were used for fat extraction. This was to make the fat more available for extraction. The samples were wrapped in filter paper and held with a clip in the extraction unit in which a weighed flask containing 50ML of petroleum ether (60-90°C) was attached while on the heating plate. The extractor was connected to a reflux condenser on a steam bath for 3 hours. The petroleum ether extract was evaporated to dryness at 100°C for 5 minutes. The flasks were cooled in the desiccator and weighed. Extractable fat was calculated using the equation (AOAC, 2006);

$$\text{\% fat} = \frac{\text{Weight (g) of flask + fat - Weight of flask without fat X 100}}{\text{Weight (G) of sample before drying}}$$

(iv) Determination of Ash

Six crucibles were washed and placed in the oven for 5 minutes. The crucibles were removed, cooled in the desiccators for 1hour and weighed. 5g of the sample was weighed into each crucible, placed on a hot plate under a fume hood and the temperature was slowly increased until smoking ceased and the samples became completely charred. The crucibles were inside the muffle furnace and ashed overnight at 550°C.The crucibles were removed from the furnace and placed in the desiccators for like an hour. When cooled to room temperature, each crucible plus ash was weighed and the weight of ash calculated (AOAC, 2006) as follows;

$$\text{\%ASH} = \frac{\text{Weight of crucible + Ash sample – weight of crucible}}{\text{Weight of sample}} \times \frac{100}{1}$$

(v) **Determination of Total Available Carbohydrate (TAC)**

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A gram of the sample was weighed and transferred into a graduated 100ML, stoppered measuring cylinder.10ML of water was added and stirred with a glass rod to dispense the sample thoroughly.13ML of 52% perchloric acid reagent was added using a measuring cylinder and constantly stirred with a glass rod for 20minutes.Samples were noticed to digest by forming slightly thick slurry. The glass rod was washed down with water and the content made up to 100ML. It was mixed and filtered into a 250ML graduated flask. The measuring cylinder was rinsed with water and transferred into the graduation flask, made up to mark and thoroughly mixed.10ML of the extracted sample was diluted to100ML with water and 1MLof the diluted filtrate pipette into a test tube. Blank and glucose standards in duplicates were prepared and anthrone reagent rapidly pipetted into all tubes, stoppered and content were thoroughly mixed. The tubes were placed in a boiling water bath for exactly 12 minutes, after which they were cooled to room temperature. The solution was transferred to 1cm glass cuvettes and the absorbance of the sample and standards reads at 630nm against the black reagent using a spectrophotometer. The total available carbohydrate (TAC) as percent glucose was calculated using the equation (James 1995);

$$\text{TAC(as\% glucose)} = \frac{\text{25x absorbance of dilute sample}}{\text{Absorbance of dilute standards x weight (g) of sample}}$$

(vi) **Determination of Crude Fibre**

This was calculated by the difference method. The percentage of all other parameters was calculated and the sum subtracted from 100. The difference became the percentage crude fibre (AOAC 2006).

STATISTICAL ANALYSIS

The obtained microbiological and proximate composition data are analyzed using descriptive statistics. The emphasis on this work is on descriptive analysis rather than hypothesis testing and p-values.

3. RESULT OF FINDINGS

MICROBIAL COUNT OF THE SKIN, MOUTH AND THE GUT.

The total Heterotrophic bacteria count, Coliform and Fungal of the skin, gut and the mouth are as follows:

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Table 1: showing the mean values of the total Heterotrophic count of bacterial and Fungal from the skin, gut and the mouth are as follows:

COLONIES	Gut (CFU/G)	Skin (CFU/G)	Mouth (CFU/G)
Total bacteria count	$6.6x10^{7}$	$5.8x10^{6}$	$5.6x10^{6}$
Total fungi count	$5.5x10^{7}$	$5.2x10^{6}$	$4.5x10^{6}$

IDENTIFICATION OF ORGANISMS FROM ISOLATION

Various bacteria and fungi isolated from the samples; *Vibrio parahaemolyticus., Vibrio cholera. Shigella spp., Salmonella spp., Escherichia coli.,Staphylococcus spp., Bacillus spp., Klebsiella spp., Pseudomonas spp.,* and also fungal species are*; yeast spp., Aspergillus spp., Penicillium spp., Candida spp., Cryptococcus species.* They were all classified based on their morphological and biochemical characteristics of Cowan and Steel (1966) and Buchanan Gibbons (1974).

OUTCOME OF PROXIMATE COMPOSITION

The nutritional composition of edible frog is given in Figure 1. The analysis performed on this frog is given as: protein (45.06%), moisture (40.27%), ash (5.70%), carbohydrate (3.00%), fat (2.55%) and fibre (3.42%).

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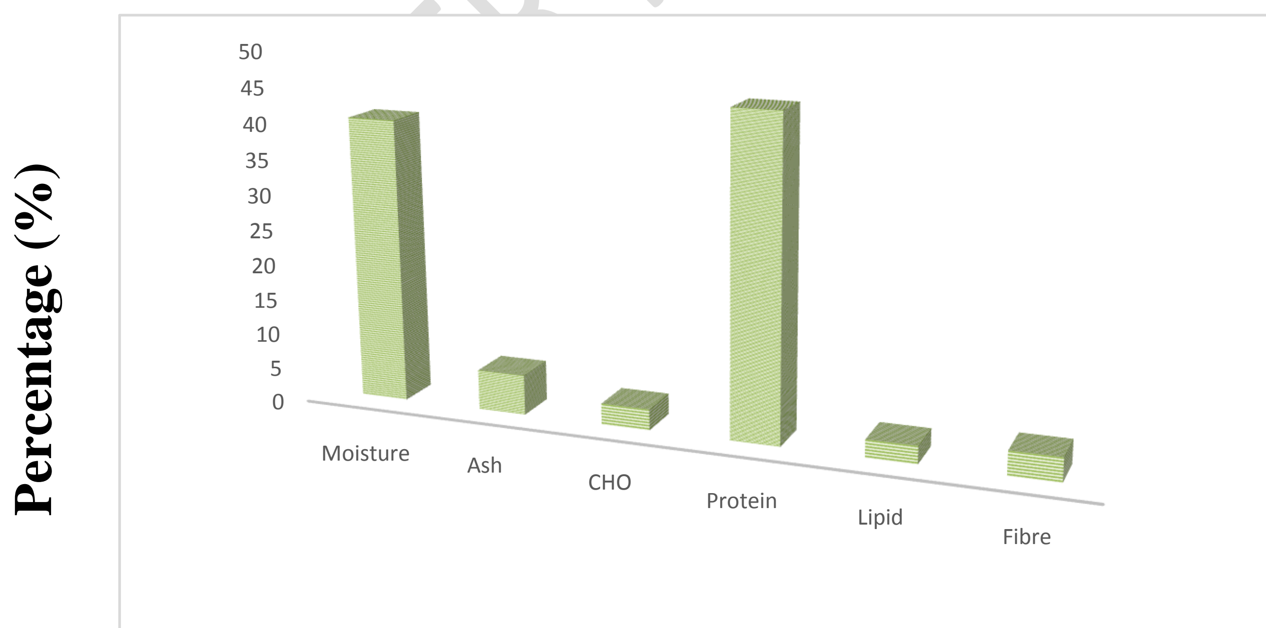

Figure 1. Nutritional composition of edible frog during proximate analysis

Table 2: showing list of tentative bacterial isolate obtained from the mouth, gut and skin of the frog after using different selective/ differential media and biochemical analysis are as follows:

Mouth	Gut	Skin
Vibrio parahaemolyticus	*Escherichia coli*,	*Bacillus spp*
Escherichia coli,	*Vibrio spp*	*Escherichia coli*
Salmonella spp.,	*Klebsiella spp*	*Salmonella*spp
Bacillus spp.,	*Salmonella spp*	*Klebsiellaspp*
Pseudomonas spp.,	*Bacillus spp*	*Vibriospp*
Vibrio cholerae	*Shigella spp*	*Pseudomonasspp*
Staphylococcus species.	*Staphylococcus spp.,*	*Shigellaspp*
	Pseudomonas spp.	*Staphylococcusspp*

Table 3: showing list of fungal species isolated from the frog mouth, gut and skin. From the mouth a total of three (3) genera were isolated, also a total of two (2) from the gut and three (3)from the skin respectively.

Mouth	Gut	Skin
Penicillium spp	*Candida spp*	*Aspergillus niger*
Aspergillus niger	*Aspergillus spp.*	*Aspergillus terreus*
Cryptococcus spp.		*Candida spp.*

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The figure showing the microbial load found in the mouth, skin and gut during first samples collection on different media

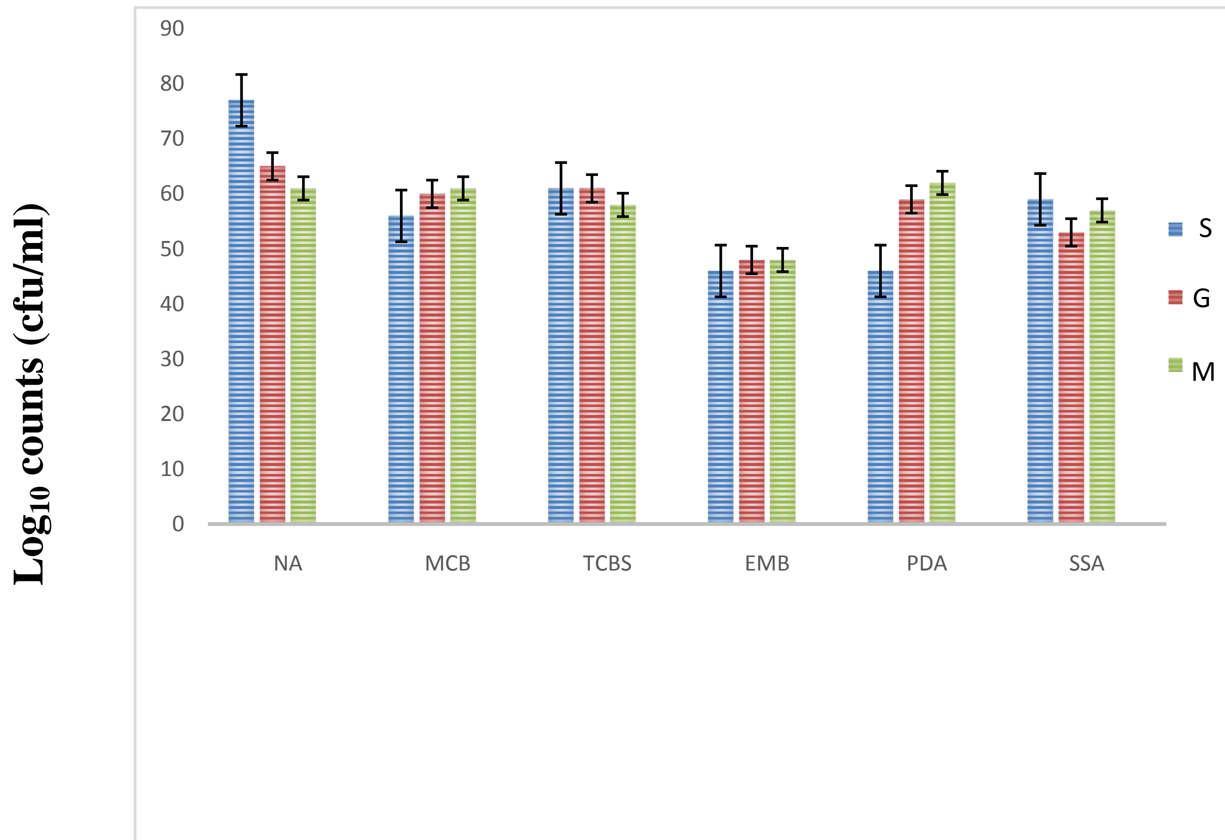

Fig.2: Microbial load in the mouth, skin and gut of the samples during first collection

KEYWORDS : G = GUT,S = SKIN, M = MOUTH

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The figure showing the microbial load found in the mouth, skin and gut during second samples collection on different media

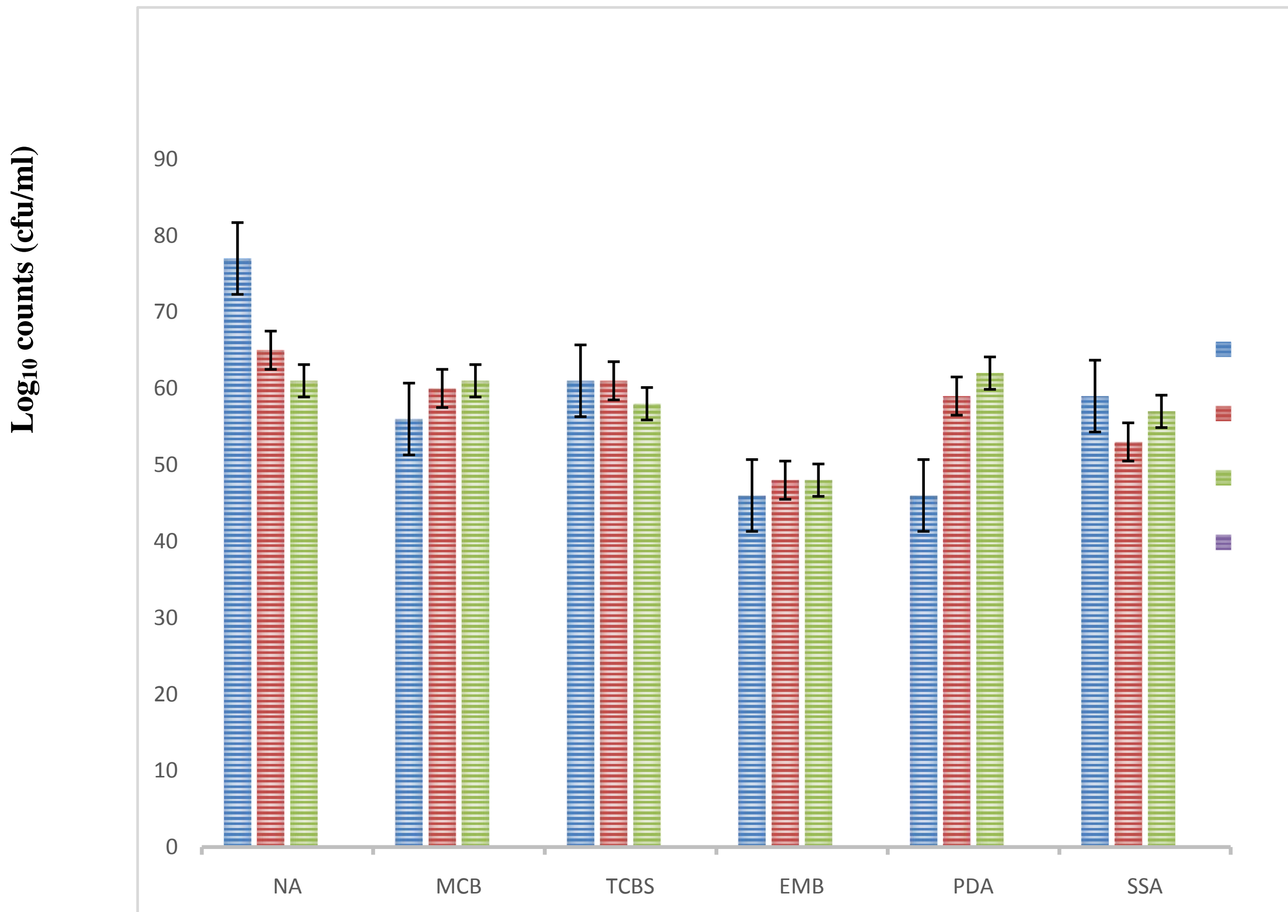

Figure 3 Microbial load in the mouth, skin and gut during second sample collection

KEYWORDS : G = GUT, S = SKIN,M = MOUTH

The figure showing the microbial load found in the mouth, skin and gut during third samples collection on different media

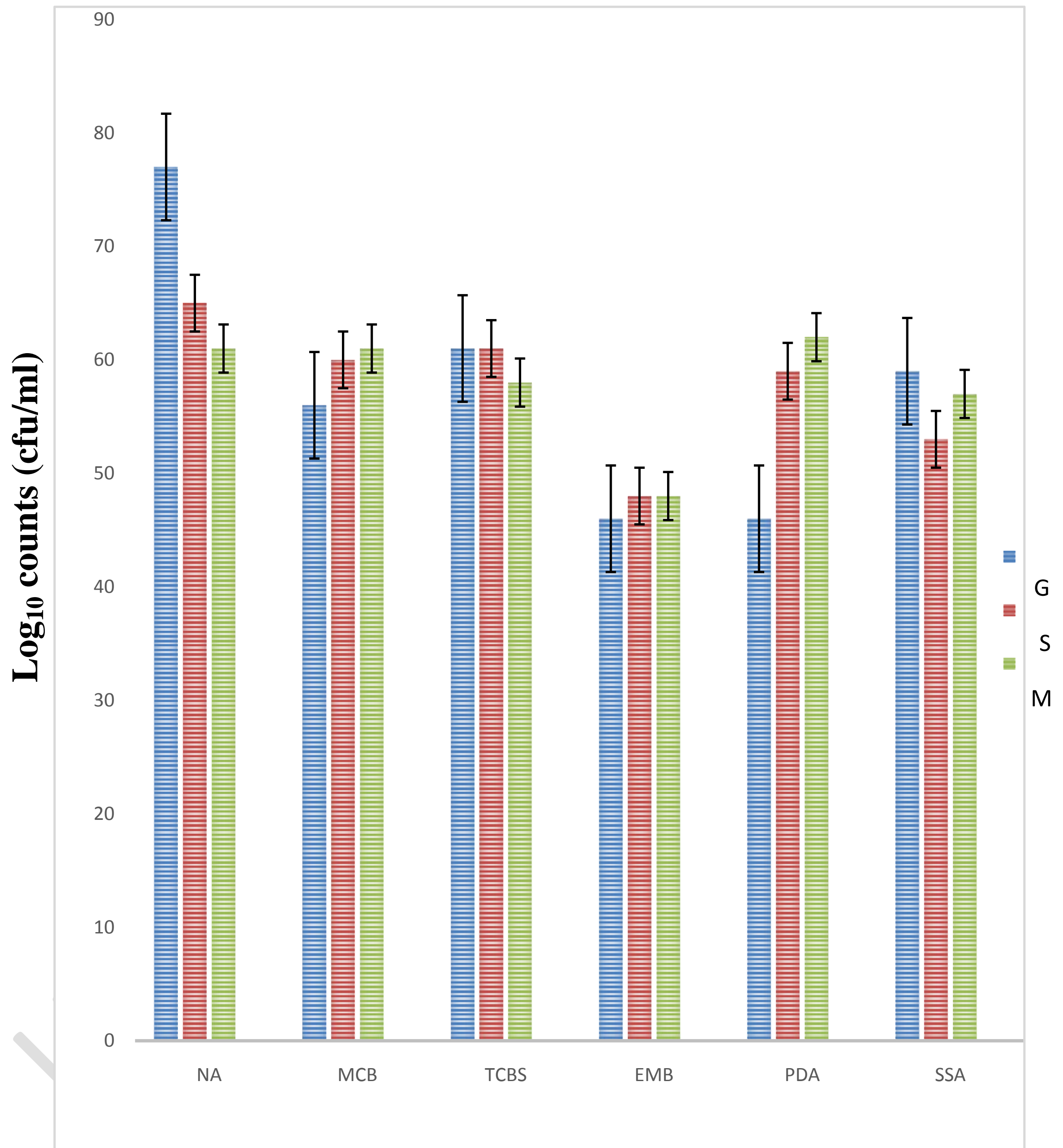

Fig.4: Microbial load in the mouth, skin and gut during third samples collection

KEYWORD: G = GUT, S = SKIN, M = MOUTH

4. DISCUSSION

The research done on this meat(edible frog) indicates the total bacteria count (total heterotrophic count) of 6.6x10^7cfu/g while a total was 5.5x10^7cfu/gisolated from the gut; 5.8x10^6cfu/g total bacteria count and total fungal count of 5.2x10^6cfu/gwas from the skin while 5.6x10^6cfu/gand 4.5x10^6cfu/gtotal bacteria and fungal count respectively from the mouth.

Based on the analysis, the gut had the highest microbial load, while the skin and mouth were less, the skin also harboured greater microbial diversity and this may be due to the soil environment where these organisms live, and these results are similar to the previous study done by Douglas and Amuzie (2017) who isolated more microbial load in the gut of Hoplobatrachus occipitalis (Frog Anura) than other part of the frog. While the total fungal count was less compared to that of bacteria. This is because the total fungal count is just a component of the medium, while the total bacteria count (total heterotrophic bacteria count) is gotten from all other media.

The media used were selective/differential to get the actual organisms associated with the meat (frog). In the media that were used, the highest growth was observed in Nutrient agar(NA) and Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar. Other media used were potato dextrose agar (PDA),mac-conkey ager (MCA), Salmonella-Shigella agar (SSA) and Eosin methylene blue. While the least were observed on Eosin methylene blue agar (EMBA). Nutrient agar plate has the higher number of growth because it is a universal media ,that is a general purpose medium that support the growth of a wide range of non-fastidious organism (organisms that has a complex nutritional requirement. It could also be due to the fact the various agar are composed of different sugars and similar findings were also observed by (Rygaard *et al*., 2017; Tamaki *et al*., 2009), which also observed that solidifying agents and composition of sugars in agar may support the growth of specific phylotypes of microorganisms. Also, tcbs having a large number of *Vibrio spp*., growing on the plate (tcbs) it is because *Vibrio* are autochthonous to aquatic animals, which increases the risk of the meat to public health, as well as the incidence as noted in work on *Pelophylax ridibundus*, by (Austin and Austin, 2007) . The presence of *Escherichia . coli*, *Salmonella spp*., and *Klebsiella*, which was also prevalent in work done by Kia *et al*., (2018) on *Hoplobatrachus spp*, may come as a result of animal or human deposits, which indicates fecal material found in the meat making it a great risk and economic loss, to the public because the rates are

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increasing day by day due to economic crisis in the country especially when the meat is not properly cooked or roasted. Although most of these organisms are normal flora of the frog, it becomes opportunistic to humans when it is not properly prepared or processed.

Bacterial isolates identified were members of both the gram positive (+ve) and gram negative (-ve) groups, which were also common flora/ inhabitants of the soil and water environments. However, more gram negative bacteria were isolated from the frog than gram positive bacteria. This observation was also made by Douglas (2017) who isolated more Gram negative than Gram positive bacteria from frogs (Anura). This raises great concern for public health as the Gram negative are proven to be more resistive to anti-bacterial interventions as supported by Breijyeh*et al*., (2020). Various bacteria and fungi were isolated from the samples. The bacteria samples are: *Vibrio parahaemolyticus, Vibrio cholera, Shigella spp., Salmonella, Escherichia coli, Staphylococcus spp., Bacillus spp., Klebsiella spp., Pseudomonas spp*., and also fungal species are: yeast spp., *Aspergillus spp., Penicillium spp., Candida spp., Cryptococcusspp*. They were all classified based on the morphological and biochemical characteristics of Cowan and Steel (1966) and Buchanan Gibbons (1974). Based on some of the organisms isolated from the samples, the meat (frog) indicates some threat or danger to the consumers, especially when it is not properly handled or prepared (cooked) before eating. Certain organisms, including *Escherichia.coli, Salmonella spp., Klebsiella*, and *Pseudomonas*, pose potential risks to consumers, while some fungi, such as *Candida species, Aspergillus species*, and *Cryptococcusspecies*, can also be detrimental to health, especially as these organism could result in interractions that affect the gut of human and ultimately diseases (Azzam *et al*., 2020; Jenkinson and Douglas, 20020). It is worth noting that some of these organisms are naturally present in the frogs as part of their normal flora. However, when the meat is adequately cooked or roasted, the pathogens are effectively destroyed or eliminated due to heat treatment.

The proximate composition Analysis carried out indicates Protein (45.06%), Ash (5.70%), Carbohydrate (3.00%), Moisture (40.27%), Lipid (2.55%) and Fibre (3.42%). The analysis indicates higher protein in the meat. Frogs contain a high protein content compared to some other animal meat. For example, a research that was carried out on *Rhynchophorusphoenicis* (African palm weevil) by Omotoso and Adedire (2007) who worked on the proximate composition of the weevil shows its protein content compared was 32.71(with chitin) and 26.85(without chitin) still were less compare to that of frog meat. Hence, the frog is a very

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good source of protein. Therefore, frog meat can be recommended in food especially those lacking protein in their diet or where other animal meat are scares or expensive, since frog meat is cheap and affordable.

CONCLUSION

The microbial quality and proximate composition of the edible frog (*Pelophylax esculentus*) were analysed to determine if the frog meat is safe for human consumption and also to check the nutritional content of the meat. Based on the analysis carried on the frog meat, the meat indicates some threat to the consumer's health if the meat is not properly cooked, roasted or even handled. But also the meat can be consumed as a good source of protein because it's cheap and easy to get too. But it must be properly cooked before eating to avoid dangers to human health; because heat can destroy those pathogens. Also the nutritional value or component of the meat made it good source of food (meat) especially protein component which can be recommended in food for those that lacks protein in their food or were animal meat are in limited proportion.

RECOMMENDATION

Higher temperature should be used in cooking and roasting of the meat before consumption since heat can destroy the pathogens in the meat.

This meat should be properlyhandledespecially during washing and processingof the meat. The meat should be washed thoroughly with aclean water before cooking or roasting.

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REFERENCES

AOAC. Official Methods of Analysis, 17th Edition. Association of Official Analytical Chemist, Arlington, 2000.

AOAC. (Association of Official Analytical Chemicals) Reference Method: 920.21 Official Method of Analysis of the AOAC (W. Horwitz Editor Eighteen Edition, Washington; D. C., 2006

Anonymous.Annual Book of ASTM standards. Water and Environmental Technology, Vol.11.02 (Water11), 520-523*, ASTM, Philadelphia, PA., 1994*

Austin B, Austin DA. Bacterial fish pathogens. 4th ed. Chichester, UK: Springer-Praxis, 2007

Azzam SZ, Cayme GJ, Martinez LR. Polymicrobial interactions involving fungi and their importance for the environment and in human disease. MicrobPathog. 140:103942, 2020

Barnett HL, Hunter BB.*Illustration of Genera of Fungi Imperfecti*, 3rd edition, Burgess Publication Co Minneapolis*, 1972, pp.62-63.*

Baygar T,Ozyur N. Sensory and chemical changes in smoked frog (*Ranaesculanta*) leg during cold storage. *Journal of Animal Vte. Adv. 9(3), 2010, pp588 – 593*.

Breijyeh Z, Jubeh B, Karaman R. Resistance of Gram-Negative Bacteria to Current Antibacterial Agents and Approaches to Resolve It. Molecules. 16;25(6), 2020, pp1340.

Buchanan R, Gibbons N. Bergey's Manual of Determinative Bacteriology (8thedn). Williams and Wilkins Co.; Baltimore, U.S.A. Am j public Health 65(3), 1974, pp315.

Cowan ST, Steel KJ. Manual for Identification of Medical Bacteria. Cambridge University Press, Cambridge. U.K., 1996

Douglas SI, Amuzie CC. Microbiological Quality of Hoplobatrachus occipitalis (Amphibia, Anura) Used as Meat. *Int Journal of CurrentMicrobiol and applied Sci 6(6), 2017, pp3192-3200.*

Harrigan WF,McCance ME. Laboratory Methods in Food and Dairy Microbiology, 8th ed. Academic Press Inc., London, 7-23,1990, pp286-303.

Ho AL, Gooi CT, Pang HK. Proximate Composition and Fatty acid Profile of Anurans Meat. *Journal of Science & Technology*. 22(1), 2008, pp23 – 29.

2012.

Holt JG, Krieg NR, Sneath PHA., Staley JT, Williams ST. *Bergey's Manual of Determinative Bacteriology,* Williams and Wilkins, Baltimore, Maryland, USA, 1994.

James CJ. The Analytical Chemistry of Foods. Chapman and Hall Press, New York,1995, pp86

UNDER PEER REVIEW

Jenkinson HF, Douglas LJ. Interactions between Candida Species and Bacteria in Mixed Infections. In: Brogden KA, Guthmiller JM, editors. Polymicrobial Diseases. Washington (DC): ASM Press; Chapter 18, 2002.

Kia GSN, Benjamin EA, Ajani EO, Otolorin GR. Sokoto Journal of Veterinary Sciences, Volume 16 (2), 2018, pp75-82

Kjeldahl J. New Method for the Determination of Nitrogen.Chem.News ,48 (1240),1983, pp101–102

Larone DH. Medically Important Fungi: A guide to identification. American Society for Microbiology Press, Washington D.C., 1995

Ogbalu OK, Douglas SI. Microbiological Investigation of Selected Flies of Public Health Importance from a Waste Dump Site in Port Harcourt, Nigeria. *IOSR Journal of Pharmacy and Biological Sciences* 10, 2015, pp75-78.

Ogbalu OK, Douglas SI. Culture-Dependent Characterization of Microbes associated with Oil Palm Kernel Borer, Pachymeruscardo in the Niger Delta. *Journal Biochem Technol*. 8, 2016, pp14 – 18.

Mary EA.Macconkey agar plates protocols. http://www.asmscience.org/content/education/protocol/protocol.285, 2005

Rygaard AM, Thøgersen MS, Nielsen KF, Gram L, Bentzon-Tilia M. Effects of gelling agent and extracellular signaling molecules on the culturability of marine bacteria. Applied and Environmental Microbiology, 83(9), 2017, pp00243-17.

Tamaki H, Hanada S, Sekiguchi Y, Tanaka Y, Kamagata Y. Effect of gelling agent on colony formation in solid cultivation of microbial community in lake sediment. *Environmental Microbiology*, 11, 2009, pp1827–1834.

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