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Microbiological Content and Proximate Composition of Edible Frogs

Charity Willy-Vidona ^a and Willy Barinem Vidona ^{b*}

^a Department of Microbiology, Edo State University Uzairue, Edo State, Nigeria. ^b Department of Anatomy, Edo State University Uzairue, Edo State, Nigeria.

Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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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. Methods: Methods involved samples collected and organs of the frog harvested and required parts isolated. Selective/differential media were used for the isolation of the sample. Biochemical characterization of the microorganism and appropriate proximate composition was done. Result: Results indicate the total heterotrophic bacterial count of 7.0x10⁷cfu/g while total fungal, were 5.9x10⁷cfu/g isolated from the gut; 6.8x10⁶cfu/ml, total bacteria count and total fungal count of 5.2x10⁶cfu/ml was from the skin while 7.3x10⁶cfu/ml and 4.5x10⁶/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%),

^{*}Corresponding author: E-mail: vidonanem@gmail.com;

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" [1]. "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 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" [1].

"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" [2,3]. "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" [3].

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.

2.1 Sample Collection

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°C in the laboratory until they were needed. The same samples were collected from the same environment following the method of collection three times.

The name of the species were concluded using both selective/ differential media and biochemical testing. Five frogs were used for each sample collection.

2.2 Media Used and Preparation of Diluent

"The following selective/differential media were used for the isolation of the sample: Nutrient (NA), Mac-Conkey Agar Agar (MCA), Shigella Agar Salmonella-(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 (NaCl2) 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°C for 15minutes before they were used. Also, the glass wares were sterilized in a hot oven at 160°C for 1 hour (Allen, 2005).

2.3 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" [1]. 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 [4] was 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" [5].

(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 [5].

(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- Shiqella 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" [6,7].

(iv) Isolation of Pure Isolates

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°C until they were required for further test.

2.4 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°C until required for further use. Further identification was done based on the cultural, morphological, biochemical and gram reactions according to Cowan and Steel [8] and also Bergey's Manual of Determinative Bacteriology [9]. While fungi were identified based on their morphological and microscopic characteristics [10,11].

2.5 Proximate Composition Analysis

(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°C 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 [12];

% Moisture =Loss of Weight of sample (g) X 100 Weight of sample (g)

(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:

Nitrogen % = Titre-Bank x Normal of acid X 1.4

Weight of sample 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" [13];

% fat = Weight (g) of flask + fat - Weight of flask without fat X 100 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 1 hour 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 [13] as follows;

%ASH = Weight of crucible + Ash sample - weight of crucible	
Weight of sample	x1

(v) Determination of Total Available Carbohydrate (TAC)

"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 1ML of 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 630 nm against the black reagent using a spectrophotometer. total available The carbohydrate (TAC) as percent glucose was calculated using the equation" [14];

TAC (as% glucose) = $25 \times absorbance$ of dilute sample 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 [13].

In CFU ten-fold dilution were used.

2.6 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. RESULTS AND DISCUSSION

3.1 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.

3.2 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 [15].

3.3 Outcome of Proximate Composition

The nutritional composition of edible frog is given in Fig. 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%).

The research done on this meat (edible frog) indicates the total bacteria count (total heterotrophic count) of 6.6×10^7 cfu/g while a total was 5.5×10^7 cfu/gisolated from the gut; 5.8×10^6 cfu/g total bacteria count and total fungal count of 5.2×10^6 cfu/gwas from the skin while 5.6×10^6 cfu/g and 4.5×10^6 cfu/g total 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 [1] 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 microbiological tests performed by Koffi et al. [16] in Côte d'Ivoire showed of the presence of Total Aerobic Mesophilic flora, staphylococci, total coliforms, Escherichia coli as well as Salmonella sp. at respective averages of 4.8.106 CFU/g, 5.105 CFU/g, 1.23.106 1.79.104 CFU/g, 4.97.104 CFU/g as compared to our work shown in Fig. 2. This poses additional danger of hastening the microbial decay process if not well preserved.

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Gut (CFU/G) Skin (CFU/G) Mouth (CFU/G) Colonies Total bacteria count 6.6x10⁷ 5.8x10⁶ 5.6x10⁶ Total fungi count 5.5x10⁷ 5.2x10⁶ 4.5x10⁶ 50 45 40 35 Percentage (%) 30 25 20 15 10 5 0 Moisture Ash сно Protein Lipid Fibre

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

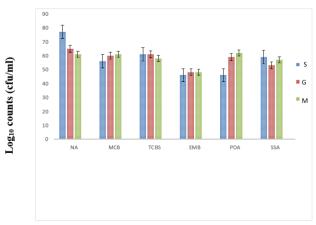
Fig. 1. Nutritional composition of edible frog during proximate analysis

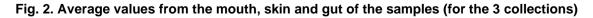
Table 2. List of tentative bacterial isolate obtained from the mouth, gut and skin of the frog after using different selective/ differential media and biochemical analysis are as follows

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	Klebsiella spp
Pseudomonas spp.,	Bacillus spp	Vibrio spp
Vibrio cholerae	Shigella spp	Pseudomonas spp
Staphylococcus species.	Staphylococcus spp.,	Shigella spp
	Pseudomonas spp.	Staphylococcus spp

Table 3. 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.





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 mac-conkey ager agar (PDA), (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 of non-fastidious wide range 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 [17,18], 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 [19]. The presence of Escherichia . coli, Salmonella spp., and Klebsiella, which was also prevalent in work done by Kia et al. [20] 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 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 [1] 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. [21]. Various bacteria and fungi were isolated from the samples. The bacteria samples are: Vibrio parahaemolyticus, Vibrio cholera, Shigella spp., Salmonella, Escherichia

Staphylococcus SDD.. coli. Bacillus spp.. Klebsiella spp., Pseudomonas spp., and also fungal species are: yeast spp., Aspergillus spp., Penicillium spp., Candida spp., Cryptococcus spp. They were all classified based on the morphological and biochemical characteristics of Cowan and Steel (1966) and Buchanan Gibbons [15]. 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 Cryptococcus species, can also be detrimental to health, especially as these organism could result in interactions that affect the gut of human and ultimately diseases [22] (Jenkinson and Douglas, 2020). Qiongping et al. [23] recently observed a perfluoroalkvl significant concentration of substances (PFASs) in wild frogs which poses threat to humans upon consumption but equally stated that steaming would be the best cooking methods for safe consumption. 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 Rhvnchophorus phoenicis (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. The amount of meat contained in the frog also varies from parts to another as seen in work done by Zhu et al. [24] where they showed that the insoluble protein content in the fore-chest meat was higher than that in the thigh meat and calf meat, but the salt-soluble protein fraction was the most abundant in thigh meat. Hatutale [25] did a work in Namibia and established that frog meat extracts exhibited antioxidant activity with the highest reducing power absorbance of 0.98±0.66 at 700nm which therefore very useful for health. Hence, the frog is a very 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 [26-28].

4. 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 of which we recommend minimal safe consumption only in its dried form. 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.

5. 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 properly handled especially during washing and processing of the meat. The meat should be washed thoroughly with a clean water before cooking or roasting.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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