Determining the bacterial content of fresh and cooked sea cucumbers sold for food consumption in Panobolon Island, Nueva Valencia, Guimaras

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Abstract

Bacterial contamination in commercially sold sea cucumbers is hugely understudied in Western Visayas. The study aimed to tackle the problem of bacterial contamination in commercially sold sea cucumbers in Panobolon Island, Guimaras by determining its bacterial content. The cooked and fresh sea cucumber samples used in this study were bought from the local market and were identified as the species *Holothuria scabra*, a common species of sea cucumber consumed as food in Western Visayas. Bacteria was isolated from both the fresh and cooked samples and cultured directly unto the selective media. Results showed that the bacterial content in cooked samples were more compared to the fresh samples. Both types of samples contained *Staphylococcus aureus,* while *Escherichia coli and Pseudomonas aeruginosa* were only present in cooked samples. *Salmonella spp.* was only present in fresh samples, while *Shigella spp.* was not found in any of the samples. The bacteria found in the fresh samples are known to be found in sea water and the content of the cooked samples can be attributed to the food handling of the residents.

***Keywords**: sea cucumbers, bacterial content, bacterial isolation, Public Health, food handling*

Introduction. Sea cucumbers (class *Holothuroidea*) are marine animals with leathery skin and elongated bodies containing single, branched gonads and belong to the phylum Echinodermata [1]. They are consumed as food in several parts of the world; and how they are consumed varies as some are eaten raw and some are cooked or dried [2]. In Western Visayas, sea cucumbers are also considered as a delicacy and are commonly caught and sold in many coastal communities, such as that of Banate in Northern Iloilo and Panobolon Island in Guimaras. According to the locals of the said places, they are prepared by either boiling, grilling, or frying, wherein the gonads and other internal organs are specially considered as a delicacy.

However, there are several potential pathogens that are present in the marine environment that may infect the sea cucumber system [3] as they are constantly exposed to high concentration of bacteria. They obtain food by ingestion of detritus or by filtration of seawater [4] and they contain microorganisms which increase the infection of gut-associated bacteria primarily occurring in the gonads and the guts. Serving as a food commodity, the locals tend to consume the gonads and the guts of the sea cucumber, which increases the risk of infection from the pathogens that might be residing in the sea cucumber. Previous studies such as that of Omran and Allam [5] have isolated bacteria such as *Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus* from the hindgut and the foregut. Sun and Chen [6] and Farouk et al. [7], have each isolated thirty (30) bacterial strains in the gut of sea cucumber *Stichopus japonicus* and *Holothuria spp.*, respectively.

The presence of bacteria in organisms used as food can also be caused by cross-contamination, which happens when food meets contaminated surfaces, such as plates and containers, during food processing and preparation [8]. Contamination of containers may be caused by direct and indirect contact with pathogens [8] since some bacteria attach to surfaces as their means of survival in nature [9]. Some foodborne bacteria can also cause deadly diseases, such as *Salmonella spp.* that causes Salmonellosis [10].

Although there are numerous studies that determine the bacterial content of sea cucumbers as marine organisms, there are still no studies that determine the bacterial content of sea cucumbers that are used for food consumption locally in Western Visayas. The aim of this study is to identify the pathogens that are present in the internal and external body walls of both raw and cooked sea cucumbers that are being used as food, which may be harmful to humans consuming the sea cucumber

The study aims to determine the bacterial content of raw and boiled sea cucumbers. It specifically aims to:

(i) Determine the bacterial density of fresh and cooked *H. scabra* sea cucumbers

(ii) Determine the presence of selected pathogenic microorganisms in fresh and cooked *H. scabra* sea cucumbers.

(iii) Compare the bacterial density and of selected pathogenic microorganisms in fresh and cooked H. scabra sea cucumbers.

Methods. The methods used are based on the study of Omran and Allam [5] and Zhang and his colleagues [11]. Sea cucumbers from Panobolon Island, Nueva Valencia, Guimaras were collected and

dissected. Morphological identification through visual comparison spicules of the collected Sea Cucumber samples was conducted using the FAO Species Catalogue and following the methods presented by Toral-Granda [12] and Perez [13]. Sea cucumbers were then processed, and bacterial isolates were obtained from the processed samples and were identified through the morphological characteristics of the bacterial colonies. Representative colonies were also gram stained and characterized through colony morphology. The presumptive presence of selected pathogenic bacteria was examined and compared using selective media.

Collection and Transport of Sea Cucumber Samples. Presented below are the methods used for the collection and transport of sea cucumber samples.

Collection of Samples. The samples were acquired in Panobolon Island, Nueva Valencia, Guimaras. Purposive sampling and pool sampling methods were used in the acquisition of the sea cucumber samples. Six H. scabra individuals were deliberately chosen using purposive sampling, as it is the most consumed species in the community, three of which are fresh samples and three are cooked/boiled based on the cooking method of the locals. The samples were then divided into two pools: one for the fresh samples and the other for the cooked samples with three organisms each pool.

Transport of Samples. The samples from Panobolon island were then transported back to the PSHS research laboratory. The duration of the transport was approximately three hours. Cooked samples were placed in an air-tight, sealed container for transport in order to minimize the contamination of the samples. They were then refrigerated after their arrival in the laboratory to minimize spoilage.

Fresh samples were transported live in an ice box filled with seawater collected from the sampling site as a preventive measure to avoid detrimental changes in levels of temperature, oxygen, and pH, all of which might be factors in the evisceration of the organisms; given that the seawater used in laboratory conditions was taken from the site of collection. Individual sea cucumber mass to seawater volume ratio, which is one Liter per ≤ 300g of sea cucumber for a ≤ 12-hour transport, was followed as recommended by Purcell et al. [14]. Additional seawater was collected to maintain the same environment for the microflora, and for laboratory use to prevent exposure to other contaminants. The lid of the container was not fully closed to reduce the changes in temperature and to minimize the splashing out of the collected seawater. Sand was also collected on the same site and was placed inside the same container serving as a supplementary sustenance for the organisms, as marine organic matter found in it is used as food by the sea cucumbers. The collected fresh samples were transferred into an aquarium when they arrived at the laboratory, with the collected sand and seawater. Aerators were then installed to ensure oxygen circulation within the aquarium. The fresh samples were utilized or dissected within 24 hours to prevent full acclimatization of the sea cucumbers to laboratory settings.

Morphological Identification of Sea Cucumber Samples. In the Philippine Science High School - Western Visayas (PSHS - WVC) microbiology laboratory, fresh sea cucumber samples obtained were placed inside an aquarium with interiors covered with black cloth for photo documentation. Top view, bottom view, and side view of the fresh sea cucumber individuals were taken. The images obtained were compared to the image presented in the Marine Species Identification Portal which is based on the ETI World Biodiversity Database, taken originally by Cannon & Silver (1986). Also, the photos presented by KM. Al-Rashidi et al. [15] which showed the various color morphs of H. scabra were utilized for comparison purposes. This was done to see the most probable resemblance of the collected fresh sample from the organisms presented by the said identification portal through visual comparison only.

The fresh sea cucumbers were also identified through their spicules. The spicules were acquired from both the dorsal and ventral area of the fresh sea cucumbers and were viewed under the microscope. They were then photographed and identified using the taxonomic classification key presented by the Commercially Important Sea Cucumbers of the World by the FAO Species Catalogue for Fishery Purposes Number 6. Also, the photography and description and photo of H. scabra spicules presented by Massin [16] was used as a guide for species identification.

The cooked samples were subjected to visual comparison only. The comparison was made before the samples were cooked by the locals. It was made sure that the visual characteristics of the pre-cooked samples were the same that of the fresh samples.

Preparation of Samples for Bacterial Isolation. Fresh sea cucumbers samples were then placed inside a container with ice-cold water for the sedation process that lasted for 30 minutes to 1 hour, as stated in the Holothuria Lab Demonstration by J. Garcia-Arraras [17]. The ice that was used in the setup was the sea water collected on site that was frozen. After which, the samples were then dissected using sterile tools from a dissection kit. The gonads and guts were removed from the fresh samples. The fresh and cooked samples were then diced using the sterile tools and were put into sterile media bottles. They were then transported to the DOST microbiology laboratory, where the samples were ground with sterile distilled water using a homogenizer, following a ratio of 1 g sea cucumber to 9 mL distilled water. This is to follow the 1:9 ratio being the 1st dilution process. The crude aqueous extracts were then centrifuged at 4900 rpm for 11 minutes. The supernatants were further diluted and preserved aseptically until use.

Media Preparation. The methods presented below are the prescribed preparation procedures for the different media that have been used for the study.

Nutrient Agar. Nutrient agar was used in order to measure and compare the bacteria density of both fresh and cooked and to characterize the morphology of the colonies that have grown in the media. It was prepared using the standard procedure

Eosin Methylene Blue (EMB) Agar. Eosin Methylene Blue Agar was prepared as a selective media to determine the presence of enteric in the samples, specifically *Escherichia coli.* Standard preparation procedures were followed in the preparation of this media.

Mannitol Salt Agar. Mannitol Salt Agar (MSA) was used as a selective media in order to determine the presence of *Staphylococcus aureus.* It was prepared using the standard procedure.

Salmonella Shigella (SS) Agar. *Salmonella Shigella* Agar was prepared as a selective media to determine the presence of *Salmonella spp.* and *Shigella spp.* in the samples. It was prepared using the standard procedure.

MacConkey Agar. MacConkey Agar was used in the detection of *Pseudomonas aeruginosa* in the samples. it was prepared using the standard procedure.

Serial Dilution. To determine and compare the presence and abundance of bacteria in the two samples, serial dilutions of the extracts were made and grown in Nutrient Agar. A preliminary study was conducted beforehand to determine the dilutions. This was performed to determine at which concentration should samples be cultured in bacteria-specific culture media for identification. The ratio of the sea cucumber mass to distilled water is one gram of the sample and nine parts distilled water, totaling to 10 mL, constituting the first dilution. Four subsequent dilutions were conducted, however, the dilutions used only had concentrations of 1:10, $1:10^3$, and $1:10^5$ for the selective media and $1:10^2$, $1:10^4$, $1:10^6$, and $1:10^8$ for the Nutrient agar to reduce the number of colonies to form for easier colony isolation, counting, and identification. The dilutions were conducted with a ten-fold ratio (1 mL aqueous extract to 9 mL distilled water).

Bacterial Isolation and Rapid Detection. The bacteria were isolated by applying 0.1 mL of the sample and was spread on different agar plates. Samples were isolated unto nutrient agar for colony count, colony morphology, and cell morphology.

Samples were cultured unto Eosin Methylene Blue (EMB) agar which was used to indicate the presence of enteric bacteria. Gram-negative bacteria will grow in the media (EMB) and growth of *E. coli* will show metallic green coloration. Samples were also cultured unto *Salmonella Shigella* (SS) agar which will indicate the presence of *Salmonella* and *Shigella* species. *Salmonella* species would produce translucent colonies with black center while *Shigella* species would produce transparent colonies. Mannitol Salt Agar (MSA) was used to indicate the presence of *S. aureus.*

Colony Morphology and Gross Characterization. Colonies cultured in Nutrient Agar were grouped and characterized using the colony morphology chart taken from Breakwell, MacDonald, Woolverton, Smith, and Robinson [18]. They were characterized into three separate categories: form, elevation, and margin. The colonies that belong to the same group were gram stained. The bacterial colonies were then heat-fixed to a glass slide and crystal violet was applied as the primary stain for 1 minute. The slide was then rinse with distilled water. Gram Iodine was then added for 1 minute and rinsed with ethanol and followed by distilled water. The secondary stain was then applied for 1 minute and was then rinsed with distilled water after. The bacterial smear was then viewed under the microscope to confirm if it is Gram-negative or Gram-positive. If the bacterial species is Gram positive, it will retain the primary stain (crystal violet) and not take the secondary stain (safranin), causing it to look violet/purple under a microscope. If the bacterial species is Gram negative, it will lose the primary stain and take the secondary stain, causing it to appear red when viewed under a microscope.

Data Analysis. Presented below are the analysis used in the study.

Bacterial Density. The bacterial densities of the fresh and cooked sea cucumber samples were analyzed through the calculation of the total colony count by getting the mean of the total colonies grown in the Nutrient Agar. It was then multiplied to 10×10^3 as a standard for getting the number of colony-forming units per gram sample. The result from the fresh samples was then compared to the cooked samples to know which sample has a higher bacterial concentration.

Presence of Selected Pathogenic Bacteria. The presence of five selected pathogenic bacteria was evaluated through visual analysis using a microbiological guide for the identification of bacteria grown in selective media.

Safety Procedure. Proper lab procedures and use of laboratory equipment of the DOST Microbiology laboratory were practiced for the whole period of the experiment. Lab gowns, gloves, head caps, and N-98 masks were worn every time when handling the bacterial sample. A safety kit was present in the laboratory in case of spillage to prevent the spread of pathogenic organisms. After the experiment, 10% bleach-water solution were applied to the petri dishes to kill the microorganisms. The petri plates and agar were disposed on a biohazard bag.

Results and Discussion. *Sea Cucumber Identification.* The fresh sea cucumbers were photographed, and the pictures were compared to the ones found in the study of Al-Rashidi and his colleagues [9] as seen in Figure 2. The spicules of the samples were also processed and were observed through the microscope. Pictures of the spicules under the microscope were also taken and compared to the pictures in the study of Massin [12] and the FAO Species Catalogue was also used in the identification of the spicules. The Sea cucumber was identified to be that of *Holothuria scabra.*

Colony Count and Gross Characterization. Bacteria colonies grew in the Nutrient Agar of both cooked and fresh samples. Fresh samples had a lesser bacterial density compared to the cooked samples, as seen in Table 1. Fresh sample cultures were only quantifiable only at the 1×10^{-2} dilution. The cultures from the cooked samples were too numerous to be quantified as there was an overgrowth in the media. At dilutions

of 10^{-6} and 10^{-8}, there were no growth observed in both fresh and cooked sea cucumbers.

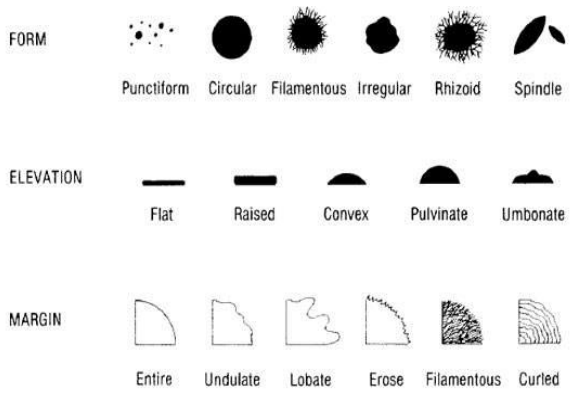

Figure 1. Colony Morphology chart guide.

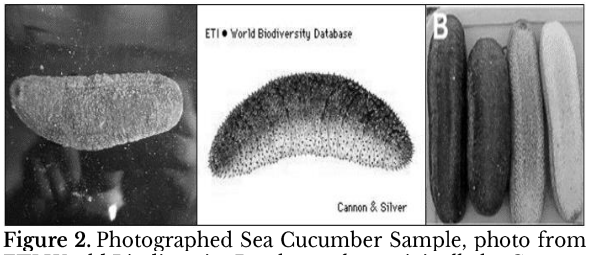

Figure 2. Photographed Sea Cucumber Sample, photo from ETI World Biodiversity Database taken originally by Cannon & Silver (1986), and a photo from the study of Al-Rashidi and his colleagues [9] respectively.

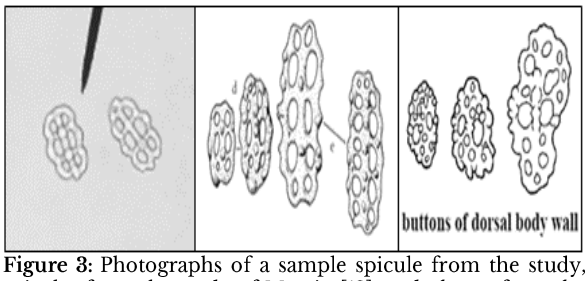

Figure 3: Photographs of a sample spicule from the study, spicules from the study of Maasin [12], and photos from the FAO Species Catalogue respectively.

Rapid Detection of Bacteria. Table 2 shows the bacteria present in the cooked and in the fresh samples. Three bacterial species are present in the cooked samples, while only 2 species can be found in the fresh samples.

Holothuria Scabra is identified to be the high-value traded sea cucumber based on the interview done with the locals and the sea cucumber identification conducted. They also revealed that *Holothuria Scabra* are being consumed as either fresh or cooked. The sea cucumbers are being prepared by the locals through boiling for their consumption.

There are several factors that could influence the results of this study, this includes environmental status, food handling, and the characteristics of certain bacteria. The results showed that the colony count of cooked samples is >2.0 x 10^{-6} which is higher than the colony count of the fresh samples which is 7.2 x 10^{-3} The results may have indicated that the samples have a high concentration of bacteria and

Table 1. Bacterial Colony Count at Different Dilutions.

	Fresh	**Cooked**
Total Colony Count	7.2 x 10^3 cfu/g sample	>2.0 x 10^6 cfu/g sample
Dilution of Sample	**Average Colony Count**	
1 x 10^{-2}	72	TNTC
1 x 10^{-4}	NG	TNTC
1 x 10^{-6}	NG	NG
1 x 10^{-8}	NG	NG

Table 2. Total Count and Bacterial Presence of Fresh and Cooked Samples

	Fresh sea cucumber samples	**Cooked sea cucumber samples**
S. aureus	+	+
E. coli	-	+
Salmonella spp.	+	-
Shigella spp.	-	-
P. aeruginosa	-	+

Legend: + indicates positive. - indicates negative.

there was some noted presence of pathogenic bacteria in both fresh and cooked samples, but this does not imply that the pathogenic bacteria have a high percentage in the bacterial flora. The strains of these bacteria were not determined, and we cannot certainly conclude that the bacteria found are indeed detrimental to the health of the consumers as some of these bacteria are commonly found in the human body.

The results of the study showed that *Staphylococcus aureus* was present in both fresh and cooked samples. Both *Escherichia coli* and *Pseudomonas aeruginosa* were exclusively found in the cooked samples while *Salmonella spp.* was only found in fresh samples. The results of previous studies, such as that of Omran & Allam (2012), showed that fresh sea cucumbers can be contaminated by *Staphylococcus aureus, Escherichia coli, Salmonella spp., Shigella spp.*, and *Pseudomonas aeruginosa. Staphylococcus aureus* is readily killed by pasteurization and cooking, but it is possible that their food handling is caused the contamination as suggested by the study of Argudin et al [19] in which Staphylococcus aureus can be transmitted by direct contact of the food with the handler's hands. The cooked samples may have come in direct contact with the hands of the person preparing it and this may have caused the sample to be contaminated with *Staphylococcus aureus.*

There was no presence of *Escherichia coli* in the fresh samples, but it was found in the cooked samples. There are several possibilities as to why the cooked samples were contaminated with *Escherichia coli.* The samples may be subjected to cross-contamination as the water and kitchen utensils used for the food preparation may be contaminated with *Escherichia coli.* According to the Center of Disease Control and Prevention, *Escherichia coli* can contaminate drinking water as well as private wells. Furthermore, the study of Dlusskaya and his colleagues [20] have found out that some strains of *Escherichia coli* can survive cooking temperatures of 71°C. The *Escherichia coli* that contaminated the cooked samples may have come from the water of the well which was used to boil the samples and it may have survived the food preparation.

Salmonella spp. was found in the fresh samples but was not present in the cooked samples. The *Salmonella spp.* may have been killed in the boiling process and there was no contamination that happened in the cooked samples. *Shigella spp.* was neither present in the fresh and cooked samples. *Pseudomonas aeruginosa* was found in the cooked samples but was not found in the fresh samples. Contamination of the cooked samples may have occurred because of improper food handling as *Pseudomonas aeruginosa* may have transmitted through any medium as it has a high adaptability and it can be found almost anywhere such as toilets, sinks, swimming pools, soap dishes, vases and dishwashers and can even survive in distilled water and disinfectants. Although there were contaminations due to the handling of the fishermen and the locals and from the environmental factors such as the water, this does not indicate error in the methods as the study is only concerned with the sea cucumber used as food. This means that handling of the fishermen are part of the food handling and we are not concerned with the way they are handling the sea cucumber.

There are several implications of having or ingesting these bacteria. Although *E. coli* is commonly found in the digestive tract of humans, some strains can cause serious disease. One of these strains can cause travelers' diarrhea and another one can cause Hemorrhagic diarrhea that can cause long term, if not fatal, health complications. *Pseudomonas aeruginosa* has been a frequent cause of nosocomial infections and this can complicate and sometimes life threatening. *Salmonella* bacteria typically live in the human intestines and the infection of these bacteria typically have no symptoms however some develop diarrhea, fever, and abdominal cramps and the infected people can recover within 8 to 72 hours. Ingesting *Shigella* can cause watery diarrhea, abdominal cramping, nausea, and vomiting. Numerous people that are infected with *Shigella* are reported to have blood in their stool. It can be fatal when there is extreme dehydration or loss of blood. *S. aureus* are usually found in the skin and are the common causes of skin infections. However, when *S. aureus* enters the bloodstream and may cause Staphylococcal sepsis. When untreated, *S. aureus* sepsis carries a mortality rate of 80%. The Methicillin-resistant *Staphylococcus aureus* is a strain that cause infections in different parts of the body but is tougher to treat since it is resistant to some commonly used antibiotics.

Error Analysis. The possible factors that can possibly affect the study are mostly because of human errors, such as: 1.) Incorrect procedures done during the transport of the sea cucumber samples from the site of acquisition to the laboratory which may cause the evisceration of the organisms; 2.) Possible spoilage of the cooked samples due to improper storage procedures and conditions, which may cause the data to be incorrect; 3.) Possible contamination of the samples from improper sterilization of the materials and equipment used for the processing of the samples; 4.) Possible contamination of the media and culture due to improper preparation procedures and sterilization; 5.) Errors that can be possibly taken from the mechanical difficulties presented by the mechanical equipment used in the experiment (e.g. Centrifure, Stomacher, etc.); and 6.) Possible errors in the morphological identification of the bacteria grown in the selective media.

Conclusion. It can be concluded that fresh sea cucumbers are potentially unsafe to eat as they are naturally contaminated with pathogenic bacteria and cooked sea cucumbers can also be unsafe to eat if not properly prepared and handled and their method of cooking is not effective.

Recommendations. If additional resources were available, the identified bacteria should be confirmed using biochemical testing and API kit. Serial dilutions done should also be done more precisely to achieve a more accurate data. Storage duration and idle time of the samples should also be reduced to avoid possible spoilage.

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