BIODIVERSITAS
Volume 25, Number 1, January 2024
Pages: 431-438

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d250150

High-throughput analysis using 16S rRNA gene of bacterial communities present in selected bivalves and gastropods species from Bayug Island, Iligan City, Philippines

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Manuscript received: 4 November 2023. Revision accepted: 31 January 2024.

Abstract. *Albarido NA, Tabugo SR. 2024. High-throughput analysis using 16S rRNA gene of bacterial communities present in selected bivalves and gastropods species from Bayug Island, Iligan City, Philippines. Biodiversitas 25: 431-438.* Seashells, which include bivalves and gastropods, have global recognition for their significant contributions to the economy, ecology, and medicine. They hold value as a food source and are highly regarded as effective biological indicators. The objective of this study is to identify the bacterial communities present in selected edible species of bivalves (*Pinctada margaritifera* Linnaeus, 1758 and *Anadara granosa* Linnaeus, 1758) and gastropods (*Canarium urceus* Linnaeus, 1758 and *Conus stercusmuscarum* Linnaeus, 1758), through high-throughput sequencing metabarcoding. Bacterial samples were collected via a swabbing technique on the surface and inside parts of selected mollusc species, which were then placed on sterilized seawater for DNA extraction. Genomic DNA was isolated from the samples, and the V3-V4 region of the 16S rRNA gene was amplified and sequenced using the Illumina MiSeq platform. Four amplicon libraries were generated, representing the two bivalve and two gastropod species in the study area. Data analysis was conducted using the Parallel Meta Suite software. Upon quality control and processing, 173,489 amplicon sequence variants (ASVs) were obtained. Within the bacterial community, the most abundant genera included *Stenotrophomonas, Vibrio, Serratia, Photobacterium,* and *Shewanella*. The assessment of alpha diversity, using the Shannon index, indicated a higher diversity in *A. granosa*. Furthermore, the analysis using the PICRUSt algorithm within the Parallel Meta Suite unveiled the involvement of specific bacteria found in the selected gastropod and bivalve species in various functions. These functions encompass protein production, xenobiotic metabolism, biodegradation, and other metabolism-related processes, supporting these organisms' ecological and physiological roles.

Keywords: Bacteria, bivalves, gastropods, parallel meta suite, seashells

INTRODUCTION

Seashells are marine mollusks encased in hard exoskeletons that serve both as structural support and protection for their bodies. According to Padhi (2021), shelled animals, including bivalves and gastropods, are commonly referred to as seashells. While some of these seashells are edible, others are not suitable for consumption. Seashells hold global significance due to their multifaceted contributions. From an ecological standpoint, bivalves and gastropods are recognized as excellent biological indicators. Any disruptions to their natural habitat or life cycles can have adverse consequences, potentially leading to depletion and posing risks to both ecosystems and human populations. Seashells are marine molluscs with a hard exoskeletons that serve as their bodies' support and protection (Sonak 2017). Seashells are known worldwide because of their vast contribution. Ecologically, bivalves and gastropods are the best biological indicators. The alteration of their natural habitat and life cycle may contribute to a harmful effect that could lead to depletion and cause harm to humans.

Seashells also carry economic importance, with many of them being utilized as a food source, as Summa et al. indicated in 2022. This is particularly relevant in light of the annual increase of 3.1% in human seafood consumption, reflecting a growing global demand for seafood, as reported in The State of World Fisheries and Aquaculture 2020. Seashells are important economically since many are used as food (Morris et al. 2018).

Apart from their economic and ecological roles, seashells offer various health benefits. They serve as valuable protein sources, and their shells have diverse applications, including decorative purposes. However, beneath their numerous positive contributions lies the potential for health-related risks associated with their extensive production. It is crucial to evaluate edible seashells as potential toxins vectors and establish surveillance measures that protect public health and ensure seafood quality, as Asakawa et al. emphasized in 2015. Seashells are known to have many health benefits as well; they are one of the best protein sources, and the shells are used for various purposes, including decorations. But behind their positive contribution is the risk of health-related incidents caused by their vast production. It is essential to assess edible seashells that are vectors of toxins and provide surveillance to protect public health and ensure seafood quality (Asakawa et al. 2015).

Globally, molluscs comprise more than 100,000 species (Rosenberg 2014) and around 22,000 species are believed to be found in the Philippines, according to the Philippine Biodiversity Conservation Priority Setting Program of 2002. Hence, morphological characters, including seashells, are regularly used for the taxonomical identification of different seafood species (Fernandes et al. 2020). But sometimes, this is difficult or impossible since these are often removed, altered, or destroyed following the species' process, storage, and transport. As an alternative, DNA-based methods have proven effective for accurate species identification and have been frequently used to authenticate some seashells and other obstructive species (Barendse et al. 2019).

DNA-based methods refer to techniques and technologies that involve the analysis, manipulation, or utilization of DNA (Deoxyribonucleic Acid), the genetic material found in living organisms. These methods have diverse applications in various scientific, medical, forensic, and biotechnological fields. Polymerase Chain Reaction (PCR), DNA Sequencing, DNA Fingerprinting, and DNA Barcoding, are some of these. DNA-based methods have transformed various scientific disciplines, contributing to advancements in medicine, agriculture, biotechnology, and our understanding of the natural world.

High-throughput sequencing platforms enable the rapid sequencing of DNA (Deiner et al. 2017). DNA metabarcoding offers a powerful molecular tool capable of non-invasively identifying and surveying species at higher taxonomic resolution than the traditional method (Bailet et al. 2020). DNA metabarcoding techniques can efficiently reveal the presence of target species at low population densities and are easy to deploy at large spatial scales (Roussel et al. 2015). It is possible to detect, acknowledge, and document bacterial communities on edible seashells and other species using this.

Bacteria are tiny single-celled organisms that are found almost everywhere on Earth: soil, rock, oceans and even arctic snow, and are vital to the planet's ecosystems. Bacteria have evolved numerous strategies to effectively adapt to environmental changes (Kloska et al. 2020). They serve as mutualists and pathogens for larger species and critical components of food webs and nutrient cycles (Farrell et al. 2019). Relatively few bacteria are parasites or pathogens that cause disease in animals, plants, and even to humans. Some bacteria cause food spoilage and crop damage, but others are incredibly useful. The primary purpose of this study is to identify the bacteria present in edible seashells through DNA metabarcoding to serve as baseline data for future studies. This will also help assess the safety of food consumption of many edible sea shells.

MATERIALS AND METHODS

Study area

The study area was situated in Bayug Island, Iligan City, Philippines. Bayug Island is a sitio within Barangay Hinaplanon positioned 3.7 km, Northeast of the Poblacion. Located approximately 8°15'30" North and 124°14'56" East, it is recognized as one of the reforested mangrove areas within Iligan Bay (Figure 1). Prior informed consent and courtesy calls were accomplished before samples were obtained.

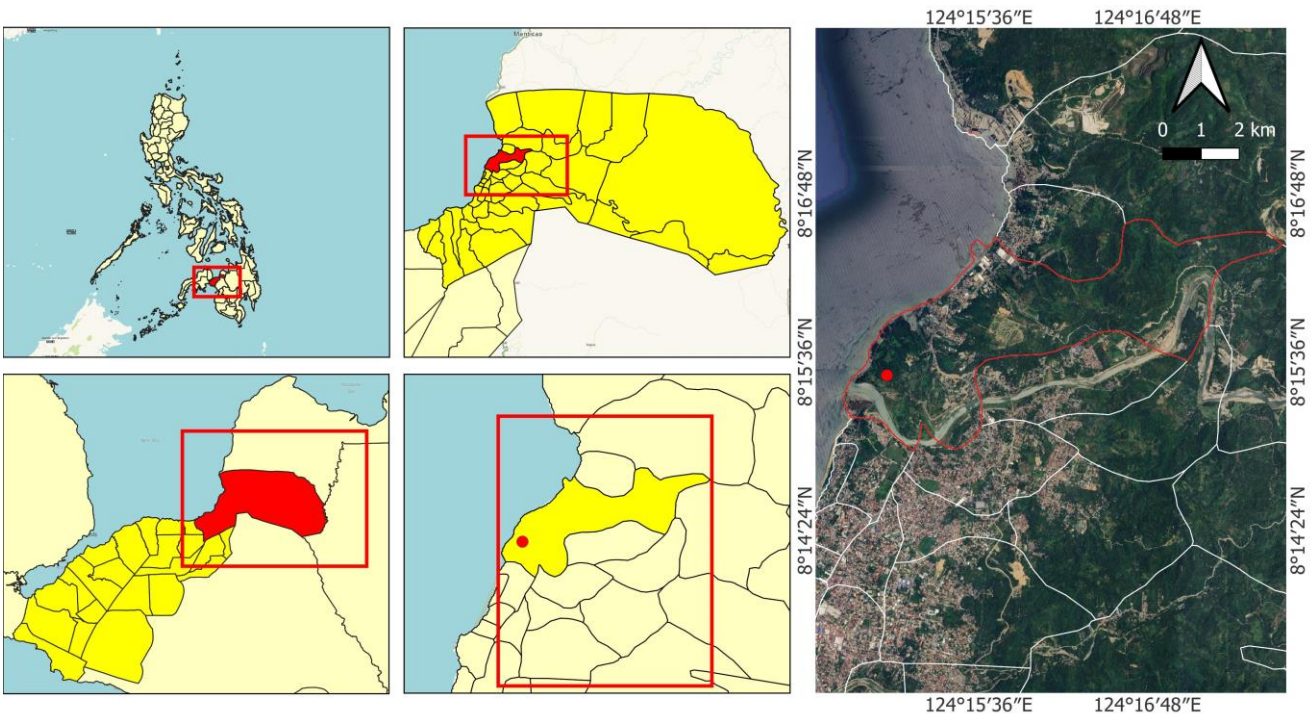

Figure 1. Map of the study area: Bayug Island, Iligan City, Lanao del Norte, Philippines

Collection of samples

Seashells that were seemingly healthy were collected in the coastal area of Bayug Island through opportunistic sampling/random handpicking. Only edible species of gastropods and bivalves were collected. These seashells were the ones suggested by the locals because these were the most popular species they glean and eat. Identification was verified through photographic guides, using taxonomic keys and consultation of experts. Also, water parameters such as temperature, pH, and salinity were recorded in situ. Collected species of bivalves were labeled as B1 and B2, while gastropods were labeled as G1 and G2 (Figure 2).

DNA extraction, amplification, and MiSeq sequencing

A ten-litre water sample was collected and brought to the Molecular Systematics and Conservation Genomics Laboratory, Center for Biodiversity Studies and Conservation, Premier Research Institute of Science and Mathematics (PRISM), MSU-IIT, for sterilization and DNA extraction. A swabbing technique was used to collect bacterial samples from the surface of the inside part of the molluscs. Swabs were then dipped in sterilized seawater for DNA extraction. Swabbing was done in triplicates and then pooled later for DNA extraction. The DNA from the water samples was extracted using a HiPurA Water Purification Kit (HiMedia) following the manufacturer's protocol. Extracted DNA was assessed by gel electrophoresis in Certified Molecular Biology Agarose gel (BIO-RAD) in 1 x TBE buffer using the Cleaver Scientific electrophoresis system (MSMINIONE). Gels were dyed with GelGreen (CA, USA) (10,000x in water). Afterwards, samples were sent to Macrogen, South Korea for Metagenome Custom Amplicon Sequencing. The V3-V4 region of the 16S rRNA gene was amplified and sequenced using the synthesized primer set Bakt_341F-long AATGATACGGCGACCACCGAGATCTACAC [index1]TCGTCGGCAGCGTCAGATGTGTATAAGAGA CAGCCTACGGGNGGCWGCAG and Bakt_805R-long CAAGCAGAAGACGGCATACGAGAT[index2]GTCTC GTGGGCTCGGAGATGTGTATAAGAGACAGGACTA CHVGGGTATCTAATCC through the Illumina MiSeq platform (Muwawa et al. 2021). This primer set was used since it produced a good DNA yield for bacteria species based on other studies. Each pooled DNA sample represents one amplicon library for further analysis.

Data preprocessing and taxonomic assignment

Paired FASTQ files were uploaded for quality check via FastQC software. Pair-end reads were merged using the Flash Length Adjustment of SHort reads (FLASH) tool. FLASH is a bioinformatics tool designed to merge paired-end reads generated by high-throughput sequencing platforms, particularly those with overlapping regions. This tool is often used in the context of Illumina sequencing, where DNA fragments are sequenced from both ends, resulting in paired-end reads (Maran 2022). Processing was done using Parallel-Meta Suite (PMS) pipeline version 3.7 available from https://github.com/qdu-bioinfo/parallel-meta-suite. Biodiversity indices were also analyzed and visualized in the pipeline. Additionally, gene families were inferred using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology with the assistance of the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) algorithm. The Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology is a classification system used to assign orthologous groups to genes based on their functional similarities. Furthermore, KEGG is a comprehensive resource for understanding the molecular functions and biological pathways of genes and their products. Furthermore, metabolic pathways were annotated using the KEGG BRITE hierarchy, a systematic approach that explores gene functions by connecting genomic data to more advanced insights into functionality (Chen et al. 2022).

RESULTS AND DISCUSSION

The Philippines serves as a sanctuary for a wide variety of marine organisms (Tabugo et al. 2013). Molluscs exhibit remarkable diversity in tropical and temperate zones, yet they are distributed across all latitudes. The muddy substrate in the area, ideal for these bottom dwellers, was the reason for their abundance (Vito 2018). Interestingly, this group of organisms, specifically the bivalves and gastropods, also serve as food and a source of important luxury goods, such as pearls.

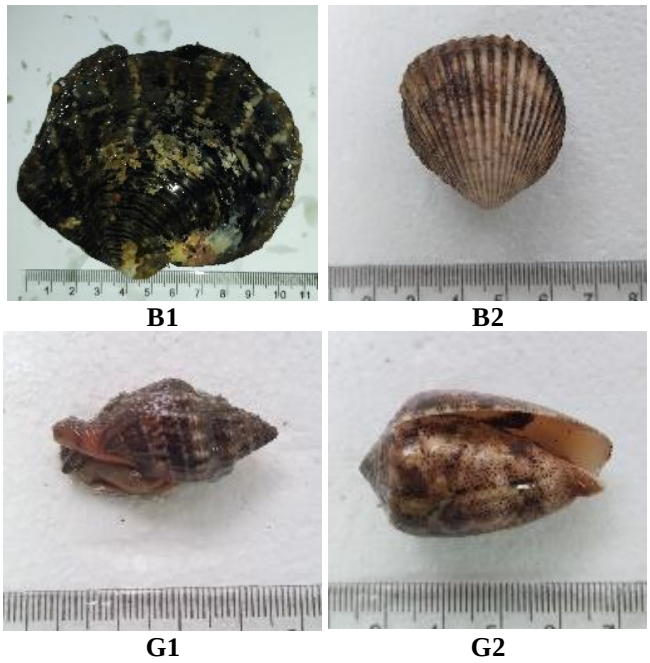

Figure 2. Photograph of representative samples: B1. *Pinctada margaritifera*; B2. *Anadara granosa*; G1. *Canarium urceus*; G2. *Conus stercusmuscarum*

Here, four economically significant seashells, composed of two species of gastropods (*Canarium urceus* Linnaeus, 1758/black-lipped conch snail and *Conus stercusmuscarum* Linnaeus, 1758/fly-specked cone) and two species of bivalves (*Pinctada margaritifera* Linnaeus, 1758/black-lipped pearl oyster and *Anadara granosa* Linnaeus, 1758/blood cockle/blood clam), were collected and used as samples for DNA metabarcoding. Results revealed that there were numerous bacterial communities present in each of the samples, but the most abundant genera include *Stenotrophomonas, Vibrio, Serratia, Photobacterium* and *Shewanella* (Table 1). Marine bacteria display a significant versatility in adapting to variations in environment and stress conditions, including temperature shifts due to seasonal changes and, even more abrupt water currents which transfer bacteria to new locations (vertically and horizontally) (Kloska et al. 2020).

High-throughput sequencing based on 16S rRNA gene explored bacterial communities. Data was analyzed using the Parallel Meta-Suite software (PMS). PMS is an easy-to-use software that allows for quick and thorough microbiome data analysis on multiple platforms. It covers a wide array of functions for data pre-processing, statistics, and visualization by state-of-the-art algorithms. A parallel computing technique optimizes the entire PMS process, allowing thousands of microbiomes to be processed quickly (Chen et al. 2022). Upon quality control and processing, a total of 173,489 raw sequence reads for the V3-V4 region of the 16S rRNA gene were obtained. There were four amplicon libraries generated, representing the 4 species of gastropods and the number of ASVs per species are the following: bivalves (*P. margaritifera*-45,414; *A. granosa*-46,875) and gastropods (*C. urceus*-45,224; *C. stercusmuscarum*-35,976) species belonging to 41 families and 76 genera, and to 43 families and 76 genera respectively. The most abundant amplicon sequence variants (ASVs) at the genus level were the *Stenotrophomonas, Vibrio, Serratia, Photobacterium* and *Shewanella*.

However, based on alpha diversity the abundance of these dominant bacterial genera varied significantly among seashells. For gastropods, the most abundant bacteria were the genus *Stenotrophomonas* for *C. ureus*, and genus *Vibrio* for the species *C. stercusmuscarum. Vibrio* species are known pathogens, and their abundance might be related to health status. Abundance can be linked to stress or disease and specific ecological niche where *Vibrio* thrives (Ina-Salwany 2019). While for the bivalve species, *Stenotrophomonas* was also the most abundant genera for species *P. margaritifera*, and the genus *Serratia* for the species *A. granosa* (Figure 3). Furthermore, the species *A. granosa* has the most diverse bacterial community. This indicates a higher diversity as based on alpha diversity using Shannon index and the Simpson index (Figure 4). *A. granosa*, a commercial blood cockles and a popular seafood, is sensitive to changes in the surrounding environment (Zarkasi et al. 2018). Its high protein and zinc content is beneficial for treating malnutrition, especially in cases of stunting in children. Packed with nutrients and a perfect growing medium for microorganisms, blood cockles (*A. granosa*) have a high potential for accumulating pollutants, such as heavy metals and microbes, due to their role as filter feeders (Ekawati and Yusmiati 2018).

Identifying organisms based on DNA has grown important within the last ten years (Stewart et al. 2017; Taberlet et al. 2018). Sampling and analyzing DNA has been dramatically facilitated by adopting high-throughput sequencing and metabarcoding techniques, transforming how we approach the inventory and monitoring of biological diversity (Bohmann et al. 2014; Deiner et al. 2017). Use of DNA metabarcoding has been especially well-suited for detecting and identifying cryptic, rare, or endangered species of plants (Craine et al. 2017), fungi (Olson et al. 2013), animals (Ishige et al. 2017; Ushio et al. 2017), and microorganisms (Richards et al. 2018). In recent years, investigations in agriculture, aquaculture, ecology, and the environment have found that 16S rRNA sequencing of bacteria is increasingly relevant for metagenomics (Zarkazi et al. 2018). This concept gained popularity primarily for its ability to explain the diversity of bacterial communities in habitats, animals, and plants.

Metagenomic analysis of DNA has the potential to completely change our understanding of ecosystem health Monitoring bacterial communities can serve as a model for tracking the spread of invasive species or infectious organisms, assessing the effects of ecological disturbances on the makeup of native communities, and understanding the response of these communities to biological contaminants (Farrell et al. 2019).

In general, using DNA metabarcoding has many practical applications, such as trophic ecology (Berry et al. 2017), plant-pollinator interactions (Vamosi et al. 2017), biological invasion detection (Borrell et al. 2017; Valentin et al. 2018; Larson et al. 2020), and air and water quality monitoring (Hurley et al. 2019; Calderón-Franco et al. 2020).

Table 1. The top five most abundant bacteria based on ASVs in seashell species through DNA metabarcoding

Seashell species		Genus of bacteria				
		Stenotrophomonas	***Photobacterium***	***Vibrio***	***Shewanella***	***Serratia***
Gastropods	*Canarium urceus*	9738	3034	4342	1972	1765
	Conus stercusmuscarum	2584	366	14282	371	515
Bivalves	*Pinctada margaritifera*	12161	4556	641	193	3732
	Anadara granosa	6849	3013	3100	84	14662
Total		31332	10969	22365	2620	20674

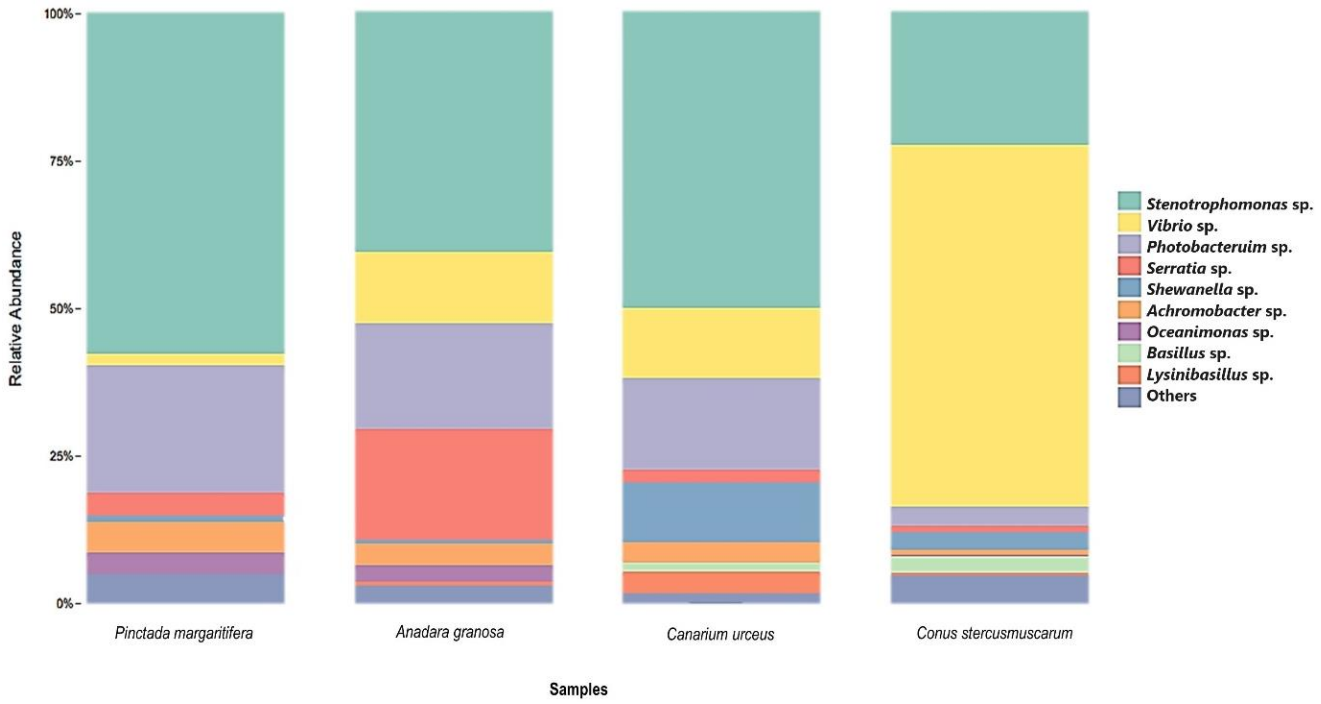

Figure 3. Relative abundance of bacterial communities present in species of gastropods and bivalves

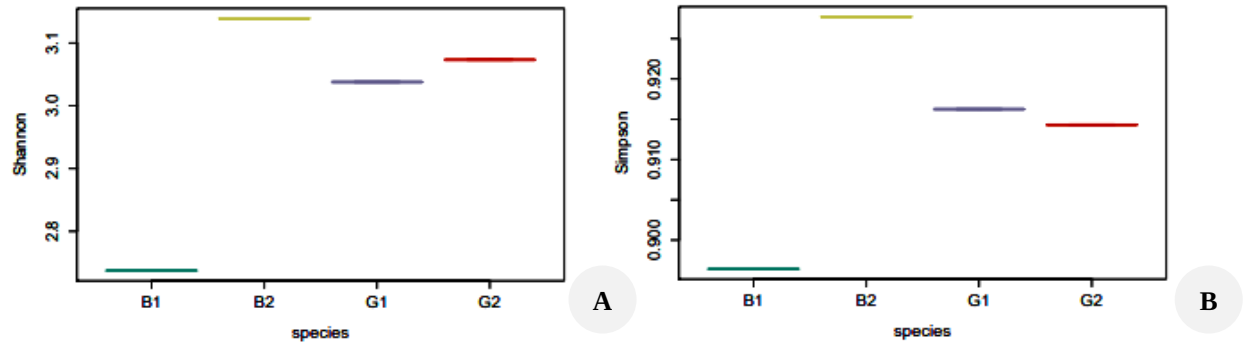

Figure 4. Alpha diversity Indices of the bacterial community present in selected bivalves and gastropods. A. Shannon Index; B. Simpson Index. B1. *Pinctada margaritifera;* B2. *Anadara granosa;* G1. *Canarium urceus;* G2. *Conus stercusmuscarum*, analyzed and visually represented from the PMS pipeline (https://github.com/qdu-bioinfo/parallel-meta-suite)

DNA metabarcoding currently exhibits its strength primarily in aquatic samples, a testament to its efficacy in deciphering the genetic diversity of organisms in aquatic environments. This preference arises from the inherent challenges associated with the fragility of organismal DNA tissue, which tends to degrade rapidly in the face of common environmental stresses (Yang and Zhang 2020). A wealth of knowledge regarding DNA degradation processes in aquatic settings has been garnered through extensive research, exemplified by studies (Barnes et al. 2014; Harrison et al. 2019).

It is important to note, however, that the limitations associated with DNA metabarcoding in non-aquatic environments are not insurmountable. Nevertheless, creative applications of DNA metabarcoding are becoming more common to identify species in terrestrial environments, even despite these technical difficulties (Cáliz et al. 2018; Walker et al. 2017; Williams et al. 2018; Sirois and Buckley 2019).

The analysis using the PICRUSt algorithm within the Parallel Meta Suite unveiled the involvement of specific bacteria found in the selected gastropod and bivalve species in various functions. These functions encompass protein production, xenobiotic metabolism, biodegradation, and other metabolism-related processes, supporting these organisms' ecological and physiological roles (Figure 5). Parallel-Meta Suite (PMS) is an easy-to-use software package that allows for fast and thorough microbiome data analysis on multiple platforms. It provides a broad range of tools for statistics, data pre-processing, and visualization by state-of-the-art algorithms. A parallel computing technique optimizes the entire PMS process, allowing thousands of microbiomes to be processed quickly (Chen et al. 2020).

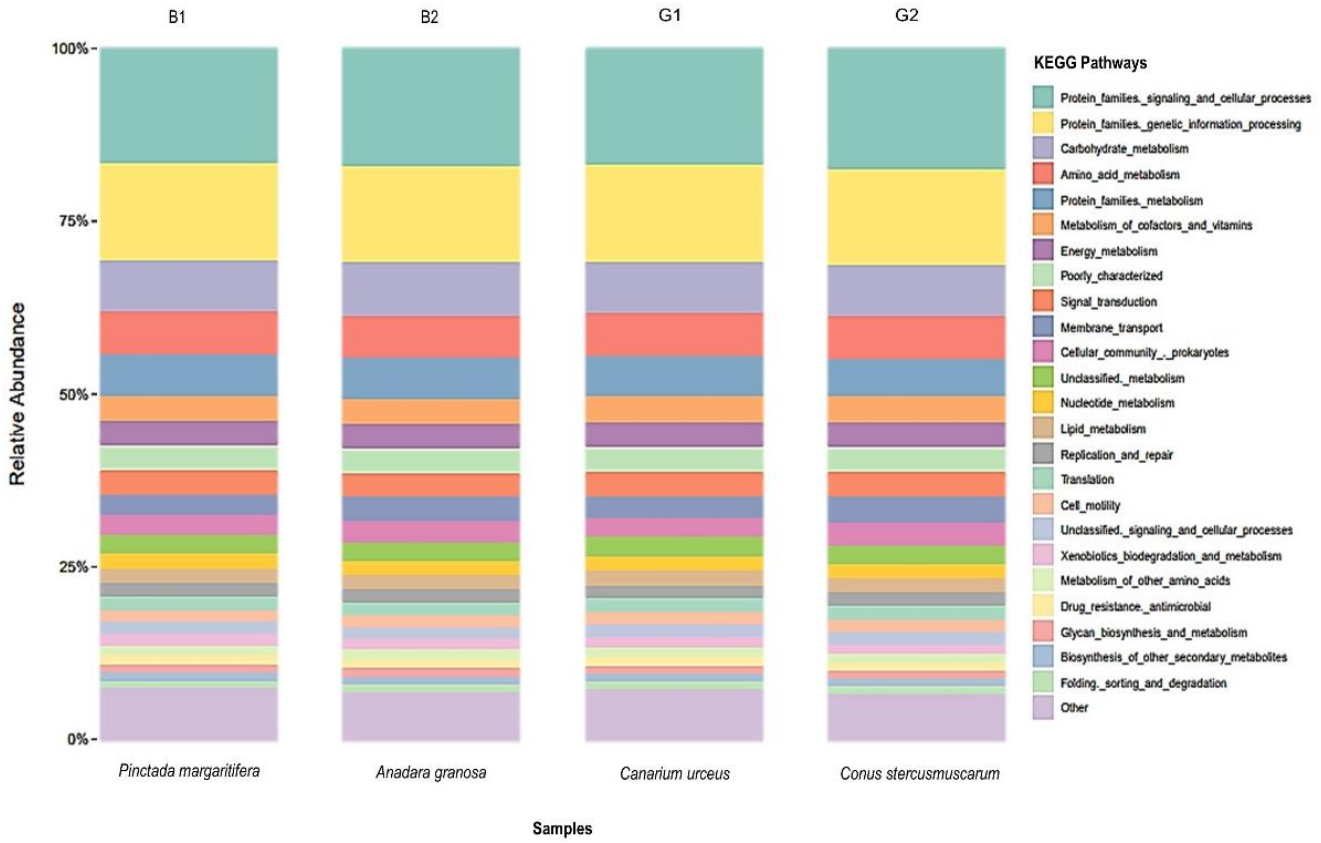

Figure 5. PICRUSt analysis of the functions of bacterial communities present in selected species of bivalves and gastropods as represented by KEGG pathways

Bacteria belonging to the Stenotrophomonas genus play a significant ecological role in both nitrogen and sulfur cycles and bioremediation processes by xenobiotic degradation to enhance crop plants' health (Ghosh et al. 2020). Furthermore, certain Stenotrophomonas species can establish beneficial relationships with plants. However, it is important to highlight that certain members of these species are now acknowledged as emerging human pathogens, leading to potentially life-threatening infections in humans. This microorganism can potentially induce respiratory and bloodstream infections, as well as a range of other infections in both healthcare settings and the broader community. It can be found in water, plant rhizospheres, animals, and food (Brooke 2021).

The *Shewanella* genus is one of the most abundant marine and freshwater bacteria. Its metabolic versatility and ability to utilize a variety of extracellular electron acceptors is a key feature in its role in the turnover of organic matter, denitrification and bioremediation (Lemaire et al. 2020). In addition, *Shewanella* species are known for their spoilage and decomposing potential even at low temperatures, which indicates that their metabolic processes are active under those conditions (Kloska et al. 2020). Toxic pollutants have made many *Shewanella* strains resistant, and it is common practice to create toxin-resistant strains in the laboratory (Baaziz et al. 2018). This characteristic, which gives these bacteria a selection advantage, is presumably made worse by the significant anthropogenic inputs of hazardous compounds in aquatic environment (Lemaire et al. 2020).

Meanwhile, *Vibrio* sp. is the causative agent of vibriosis that infects marine animals such as fish, shrimp and shellfish (Ina-Salwany 2019). It is primarily found in marine environment and is known as a common food-borne pathogen in Asia. *Vibrio* sp. is responsible for approximately 10-20% of foodborne diseases such as gastroenteritis particularly from traditional consumption of raw or undercooked seafood (Devi et al. 2019). Within the diverse array of over 70 *Vibrio* species that inhabit marine, estuarine, and freshwater environments, 12 have been identified as pathogens that affect humans (Kokashvili et al. 2015). According to Gomez-Gil et al. (2014), vibriosis is a bacterial disease that affects fish, crustaceans, molluscs, corals, and rotifers. It is mainly caused by certain species of *Vibrio* and *Photobacterium*, which are notoriously recognized as significant marine diseases.

More than 35 species of the genus *Photobacterium* have been found in aquatic environments across the globe. They exhibit various ecophysiological traits, including parasitic, symbiotic, free-living, and piezophilic lifestyles (Labella et al. 2017). Some species of this genus, including *Photobacterium rosenbergii*, *Photobacterium jeanii* and *Photobacterium sanctipauli*, cause various diseases on animal hosts, including corals, sponges, and zoanthids (Moreira et al. 2014). Subspecies of *Photobacterium damselae* have drawn much interest as newly discovered diseases for

fish, molluscs, crustaceans, and other aquatic organisms, and even for humans (Moi et al. 2017). *Photobacterium damselae* subsp. damselae is a primary pathogen of several wild- and cultured-fish species causing wound infections and haemorrhagic septicaemia. In June 2019, a local *Penaeus vannamei* Boone, 1931 culture experienced huge mortalities in Hainan Province, China. The diseased shrimp displayed evident black gills. Three bacterial strains were isolated from the hepatopancreas and gills of the diseased shrimp and identified as *Photobacterium damselae* subsp. damselae based on the sequence analysis of 16S rRNA and toxR genes (Wang et al. 2020). It is also an opportunistic human pathogen, causing necrotizing fasciitis also known as flesh-eating disease (Rivas et al. 2013).

Serratia, conversely, is a distinct class of enterobacteria with a remarkable secondary metabolism which can generate a large variety of naturally occurring bioactive compounds. However, until recently, most *Serratia* isolates were derived from infections in humans and animals, and a methodical strategy for isolating *Serratia* from natural habitats has yet to be discovered. So far, the lack of environmental isolates has limited our understanding of *Serratia*'s potential to produce new natural bioactive products and their capacity to be used in sustainable agriculture as biocontrol agents (Soenens and Imperial 2019).

Here, the findings provided an encouraging glimpse into the potential of utilizing DNA metabarcoding to assess bacterial communities associated with seashells. This approach can enhance our ability to depict these communities' structure and functionality, and can be widely adopted due to its popularity in exploring the diversity of bacterial communities across diverse ecosystems, including those linked to animals and plants. Traditional techniques, reliant on bacterial cultivation, have historically been employed to study these communities, granting access to cultivable bacteria in various habitats. Conversely, culture-independent approaches, such as 16S rRNA sequencing, permit the identification of bacteria that cannot be cultured. These non-culturable bacteria can represent more than 90% of the bacterial population, which may go unnoticed and are yet to be identified. It is feasible to detect and classify species using DNA that has been isolated, conserved, amplified, sequenced, and classed based on its sequence from environmental samples (Deiner et al. 2015).

In essence, DNA metabarcoding stands out as a non-invasive, efficient, cost-effective, and highly sensitive method, offering significant potential to enhance biodiversity monitoring in ecological studies and management endeavors across extensive spatial and temporal scales. This capability opens avenues for in-depth exploration, enabling us to delve into a more comprehensive understanding and manipulation of bacterial communities in diverse environments, encompassing both animals and plants.

ACKNOWLEDGEMENTS

We want to express our heartfelt gratitude to the Barangay Council of Bayug Island, John Arlu Bautista, Ms. Darshel Ester P. Estor, Ms. Stefenie Katrin Siblos, Christian Dave Tipudan and Ms. Manilyn Laya-og for making this paper possible. We would like to thank the Department of Science and Technology-Science Education Institute (DOST-SEI) for funding this research.

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