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Diversity and functional annotation of bacteria community associated with the epithelial surface of *Hippocampus kuda* (Yellow seahorse) and its surrounding environment

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Abstract. *Maguate NJIFS, Tabugo SRM. 2024. Diversity and functional annotation of bacteria community associated with the epithelial surface of* Hippocampus kuda *(Yellow seahorse) and its surrounding environment. Biodiversitas 25: 2230-2240.* *Hippocampus kuda*, commonly known as the Yellow seahorse, hosts various micro-organisms crucial for its well-being and interactions within its ecosystem. This research aimed to analyze and understand the bacterial community associated with *H. kuda* by employing V3-V4 16S rRNA gene amplicon sequencing on the Illumina MiSeq platform. Four distinct libraries were constructed, representing the epithelial surfaces of both male and female seahorses and samples from water and soil/sediment habitats. Following rigorous quality control and processing, 187,972 Amplicon Sequence Variants (ASVs) were identified. The predominant ASVs were attributed to genera such as *Vibrio, Roseobacter, Photobacterium, Ruegeria, Candidatus, Pseudoalteromonas, Synechococcus, Flavobacterium*, and *Altererythrobacter*, along with some unidentified genera belonging to the Proteobacteria phylum. As indicated by the Shannon index, alpha diversity analysis demonstrated the highest bacterial diversity on the epithelial surface of male seahorses (MS), with a value of 3.2363. Moreover, the functional annotation of bacterial community was conducted using the PICRUSt algorithm within the Parallel-Meta Suite (PMS) software. This analysis uncovered various functional categories, such as metabolism, genetic information processing, and cellular processing. Overall, the findings underscore the role of the microbiome on the skin and surrounding environment in influencing the growth and health of *H. kuda* seahorses. Notably, this study represents the first documentation of the bacterial community inhabiting the epithelial surface of *H. kuda*, shedding light on its significance in the ecology of these marine organisms.

Keywords: Amplicon sequencing, ASV, functional annotation, PMS

INTRODUCTION

Marine microbial communities are crucial for detecting environmental shifts important for ocean ecology and global health. These communities naturally adhere to their host, existing as either symbolic partners or free-living entities (Roychowdhury et al. 2018). Bacteria residing on marine organisms skin and gut significantly influence overall host health and survival (Coates et al. 2019; Sylvain et al. 2020). Skin serves vital functions like protection against injuries, microbial invasion (Hsu et al. 2020), immune defense, disease resistance (Gómez and Balcázar 2008), and nutrient acquisition (Tanu et al. 2012). Environmental factors like anthropogenic pressures and seasonal changes shape skin microbiota, impacting host susceptibility and mucosal immunological tolerance (Mahmud et al. 2022). Recent studies highlight how these environmental factors influence microbiota stability (Rothschild et al. 2018), and marine organisms like seahorses.

Seahorses, marine bony fish (Teleosts) categorized under the family Syngnathidae and the genus *Hippocampus*, include pipehorses, pipefishes, and sea dragons (Vincent 1996; Lourie et al. 2004). They are renowned for their medicinal and ornamental value (Hou et al. 2018). Among these species, *H. kuda*, commonly known as Yellow seahorse, holds particular interest. They are widely distributed throughout the Indo-Pacific region, including the Philippines. Regrettably, due to factors such as high demand in Traditional Chinese Medicine (TCM), habitat degradation, advancements in trawling technology, and unintentional by-catch, *H. kuda* has been identified as a vulnerable species by the International Union for Conservation of Nature (IUCN) (Koldewey and Martin-Smith 2010; Nuryanto et al. 2020). Beyond their significance in conservation efforts, seahorses are research subjects due to their distinctive microbiome composition, which can play a role in their overall health and physiological functions (Koning and Hoeksema 2021; Pappert et al. 2023).

The dermal structure of Yellow seahorses is notably distinctive due to its flame cone cells, which protrude above the epithelium and are covered by a prominent mucous cap (Ortega et al. 2021). Unlike other cells, these mucous caps have the potential to facilitate the growth of epiphytic microbes (Bereiter-Han et al. 1980). Given that seahorses primarily inhabit tropical and shallow seawaters, they are particularly susceptible to fluctuations in environmental conditions (Lourie et al. 2004; Zhao et al. 2023).

Consequently, seahorses can harbor many bacteria on their skin over time, influenced by their epithelial structure and the characteristics of their surrounding environment.

Recent research has delved into the microbial communities of seahorses to discover bioactive compounds, refine breeding techniques, enhance health outcomes, and gain insights into their physiological adaptations, given their heightened sensitivity and susceptibility to diseases (Li et al. 2015; Jiang et al. 2020; Wang et al. 2020; Zhao et al. 2023). Seahorse microbiomes have been observed to change structure and activity in response to environmental shifts, impacting host development and physiological conditions (Wahl et al. 2012; Zolti et al. 2020).

Bacteria within the microbiome confer benefits to hosts and other species by aiding in nutrient absorption, producing bioactive compounds, facilitating quorum sensing, and providing defense mechanisms against pathogens (Hacquard et al. 2015; Sansone et al. 2015; Wahlström et al. 2016; Galloway-Peña and Kontoyiannis 2020; Dechavez et al. 2022). Numerous studies have investigated various seahorse species, focusing on identifying and categorizing the diverse bacteria present, though exploration of their metabolic pathways has been limited (Li et al. 2015; Ko et al. 2016).

Further exploration is warranted to explore the microbial diversity present on the epithelial skin of *H. kuda* and its functional implications, although there are several studies conducted on different species (Ko et al. 2016; Wang et al. 2020; Ortega et al. 2021). Therefore, this study seeks to evaluate and present a thorough understanding of the microbial communities and their functional annotations associated with *H. kuda* through 16S rRNA gene amplicon sequencing. To our knowledge, this study serves as foundational data, as the initial documentation of the bacterial community found on the epithelial surface of *H. kuda*.

MATERIALS AND METHODS

Sampling site

Sampling was conducted on Bongo Island, sitio of Barangay Litayen, Parang, Maguindanao del Norte Philippines, located approximately 7°18'48" North and 124°1'50" East (Figure 1), based on local surveys of seahorse sightings in the area.

Sample collection

Prior informed consent was secured from the barangay, and a gratuitous permit was obtained from the Bureau of Fisheries and Aquatic Resources (BFAR) GP No. 0217-21 for this research. Opportunistic sampling, which entails free-diving and snorkeling methods, was employed instead of using transects, conducting random surveys across various areas during the sampling period. A total allowable number of 6 adult individuals for male and female specimens of *H. kuda* were collected under GP No. 0217-21. These samples served as voucher specimens. Also, 30 liters of water and sediment samples (50-100 g) were collected from Bongo Island, Parang, Maguindanao. Additionally, physico-chemical parameters such as surface water temperature, pH, and salinity were measured in the vicinity where seahorses were found.

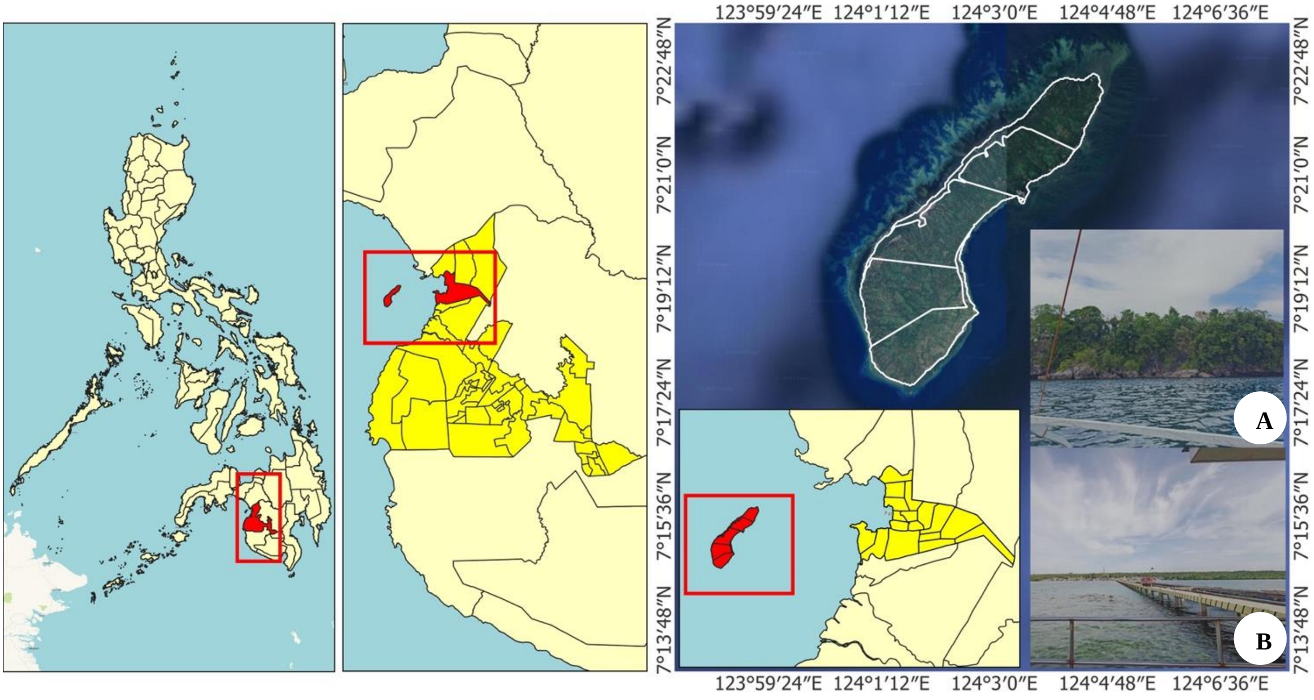

Figure 1. Map of area research in Bongo Island, Parang, Maguindanao, Philippines. Note: A. Barangay Litayen, Bongo Island site 1; B. Barangay Litayen, Bongo Island site 2

Seawater samples were filtered onsite using a sterile 0.22 µm pore-size filter membrane in a Buchner funnel. The filtered membranes were then stored in capped, sterile tubes, placed in a portable cooler, and immediately transported along with the seahorse samples to the Molecular Systematics and Conservation Genomics Laboratory, Center for Biodiversity Studies and Conservation (CBSC), Premier Research Institute of Science and Mathematics (PRISM), MSU-IIT for immediate processing. The seahorse samples underwent a washing process with sterile seawater, repeated twice (Nurul et al. 2019), to reduce any potential contaminants that could interfere with subsequent procedures, thereby ensuring the quality and accuracy of the experiments or analyses. Bacterial samples were aseptically collected from the skin mucus/epithelial surface of *H. kuda* using a conventional direct swabbing method, starting from the head down to the tail, with a sterile swab as previously described (Balcázar et al. 2010). The swab samples were then placed in a 10 mL beaker containing 2 mL of sterile Phosphate Buffer Solution (PBS) to preserve the integrity of the cells. The resulting liquid that presumably contained bacterial cells obtained from the skin was transferred to a 2-mL microcentrifuge tube and stored at -20°C until DNA extraction.

DNA extraction, amplification, MiSeq sequencing and data processing

Total bacterial genomic DNA (gDNA) was extracted from the skin of *H. kuda* and water samples using the HiPurA® MB577 Water DNA Purification Kit, while the HiPurA® MB542 Soil DNA Purification Kit (Himedia Laboratories, India) was utilized for sediment samples, following the manufacturer's instructions. The quality of the gDNA extracts from all samples was assessed using gel electrophoresis. Subsequently, the samples underwent quality check in Macrogen, Korea, before Custom Metagenome Amplicon Sequencing.

DNA was amplified using the following primers: Forward Primer (Bakt_341F-long): AATGATACGGCGA CCACCGAGATCTACACTCGTCGGCAGCGTCAGATG TGTATAAGAGACAGCCTACGGGNGGCWGCAG, Reverse Primer (Bakt_805R-long): CAAGCAGAAGAC GGCATACGAGATGTCTCTCGGGCTCGGAGATGTGT ATAAGAGACAGGACTACVGGGTATCTAATCC (Muwawa et al. 2021).

The Polymerase Chain Reaction started with an initial denaturation at 98°C for 30 seconds, followed by 10 cycles consisting of denaturation at 98°C for 10 seconds, annealing at 55°C for 10 seconds, and extension at 72°C for 30 seconds. Subsequently, an additional 25 cycles were carried out: denaturation at 98°C for 10 seconds, annealing at 65°C for 10 seconds, and extension at 72°C for 30 seconds with an expected product size between 390 to 410 bp. Sequencing was done under the Illumina MiSeq Platform.

Data analysis

FastQC software was used for quality control checks on the raw sequences. After which, the resulting pair-end reads were merged using the Flash Length Adjustment of Short Reads (FLASH) tool, a swift and accurate tool that eliminates incorrect or unreliable reads thereafter (Magoč and Salzberg 2011). The MiSeq raw reads underwent processing utilizing the Parallel-Meta Suite (PMS) pipeline version 3.7, available at (https://github.com/qdu-bioinfo/parallel-meta-suite). This pipeline is a highly efficient and interactive tool for microbiome analysis, encompassing various functionalities, including data processing, statistical analysis, and visualization. The software is an automated pipeline. Advanced algorithms within the pipeline facilitate visualization of biodiversity indices such as Shannon, Simpson, and Chao1, thereby providing insights into species relative richness and evenness (Chen et al. 2022).

Furthermore, metabolic pathways were elucidated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology via the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) algorithm. PICRUSt systematically examines gene functions by linking genetic data to more advanced functional insights (Chen et al. 2022).

RESULTS AND DISCUSSION

Physico-chemical parameters

The physico-chemical parameters were meticulously measured, revealing an average water temperature of approximately 25.5°C, a water pH level hovering around 7.2, and a salinity measuring 30 ppt. Research indicates that the marine environment is a complex and dynamic ecosystem (Fleming et al. 2019; Borja et al. 2020), wherein microbial communities are vitally influenced by various factors such as pH, temperature, salinity, dissolved oxygen, and nutrient levels, which shape their composition (Ininbergs et al. 2015; Bunse et al. 2016).

According to the analysis, although the average surface water temperature was 25.5°C and the salinity was 30 ppt, no significant relationship was observed with bacterial taxa diversity (beta-diversity). However, concerning the pH, measured at 7.2, leaning towards an alkaline condition, it is noteworthy that alkaline environments characterized by high pH levels favor the prevalence of alkaliphilic bacteria (Padan et al. 2005). Among these bacteria, representatives from the phyla Cyanobacteria, Firmicutes, and Proteobacteria are notable. The results indicate a significant presence of *Vibrio, Roseobacter, Photobacterium, Ruegeria, Candidatus, Pseudoalteromonas, Synechococcus, Flavobacterium,* and *Altererythrobacter*, across the four samples (epithelial surface of male and female seahorses, water, and sediment).

Identification of bacterial communities

Bacterial communities associated with *H. kuda* were investigated using high-throughput sequencing based on 16S rRNA genes. A total of four amplicon libraries were established, corresponding to the epithelial surface of Male (MS) and Female Seahorses (FS), Water (WS), and Soil/Sediment (SS). Following post-quality control procedures, 187,972 amplicon sequence variants (ASVs) were obtained, representing 534 families and 890 genera. Among these ASVs, the most prevalent genera included *Vibrio, Roseobacter, Photobacterium, Ruegeria, Candidatus, Pseudoalteromonas,* *Synechococcus*, *Flavobacterium*, *Altererythrobacter*, and some unidentified bacteria from the family Gammaproteobacteria (Figure 2). Most of the unidentified genera belonged to the phylum Proteobacteria, which encompasses various pathogens found in mesophilic environments such as marine sediment, freshwater, and soil (Fukuyama et al. 2020).

The Alpha-diversity indices (Shannon, Simpson, and Chao1) were computed at the genus level to assess the diversity measure as shown in Figure 3. Shannon diversity index (H′) demonstrated that the epithelial surface of the Male Seahorses (MS) had the highest bacterial diversity value of 3.2363, followed by the Water Sample (WS), the epithelial surface of the Female Seahorses (FS), and soil/sediment, having the lowest bacterial diversity of 2.218. Simpson index was 0.9240 in WS, followed by MS (0.9116), FS (0.8920), and SS (0.8001).

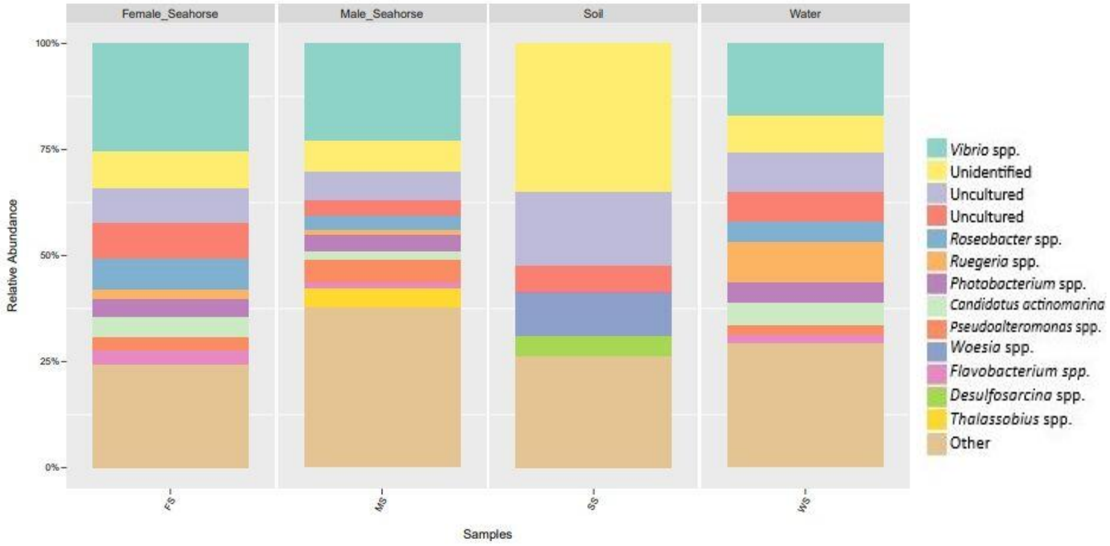

Figure 2. Relative abundance of bacterial communities between samples: Epithelial surface of Male Seahorses (MS) and Female Seahorses (FS), Water Sample (WS), and Soil/Sediment (SS)

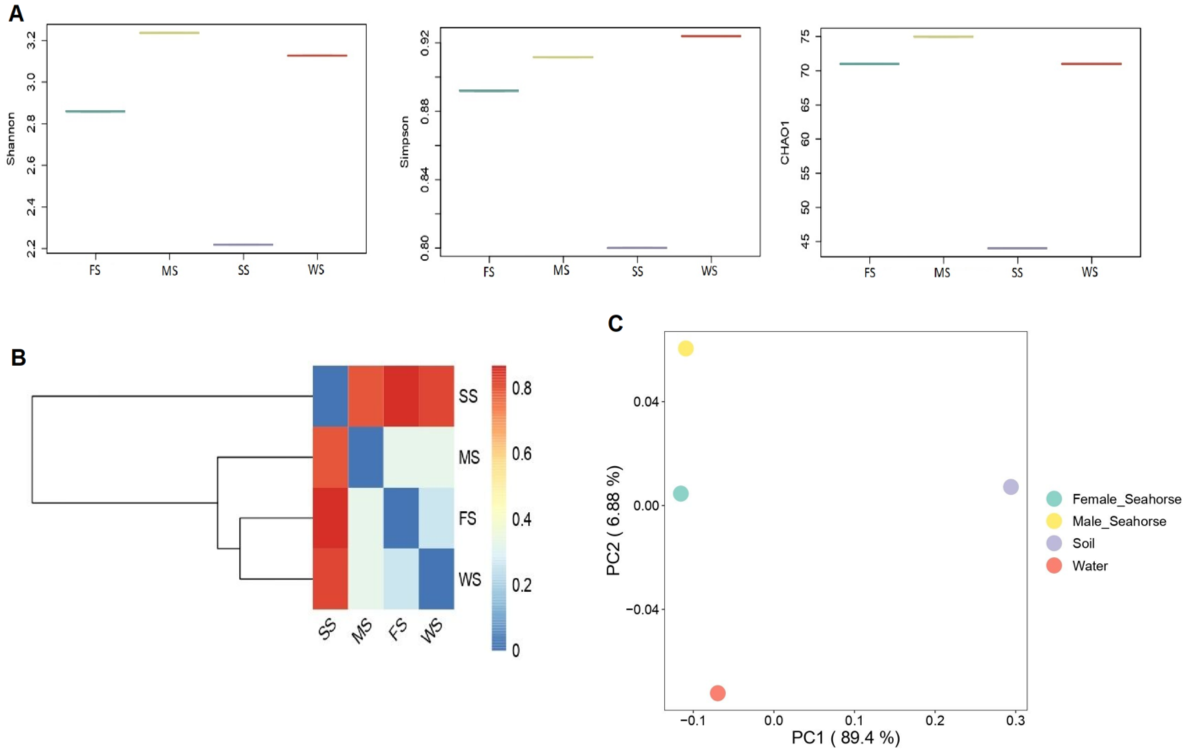

Figure 3. A. Alpha diversity indices of the epithelial surface of Male Seahorses (MS), Female Seahorses (FS), Water Sample (WS), and Soil/Sediment (SS); B. Heatmap showing the beta diversity pairwise distance matrices between samples (MS, FS, WS, SS); C. Principal component analysis illustrates the clustering of amplicon libraries in reduced dimensions derived from beta diversity analysis

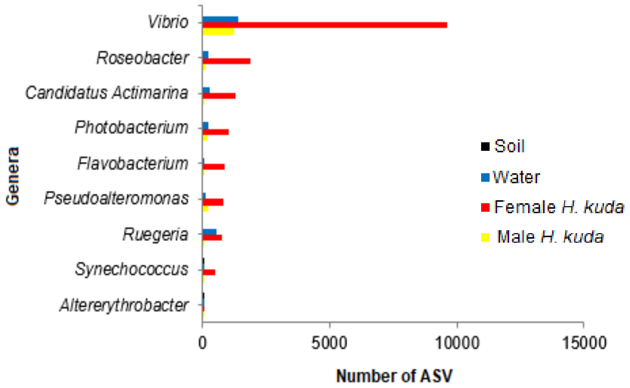

Figure 4. Amplicon Sequence Variants (ASVs) count comparison between the epithelial surface of male and female seahorses, water, and soil/sediment

Simpson's index provides insight into the relative abundance of species, while Shannon's index focuses on the diversity or richness within the community. Additionally, Chao1 is a nonparametric method for estimating species count within a community. In this study, more species were identified on the epithelial surface of Male Seahorses (MS). For evaluating beta diversity, hierarchical meta-storms were utilized to generate pairwise distance matrices, visualized as heat maps indicating high abundance on the epithelial surfaces of female and male seahorses. Moreover, Principal Component Analysis (PCA) was conducted to explore the clustering of amplicon libraries based on beta diversity automated from the pipeline. The PCA plot illustrates the separation of amplicon libraries collected from the four samples (Male Seahorses, Female Seahorses, Water Sample, Soil Sample), reducing multidimensional data to distinct clusters.

Figure 4 shows the Amplicon Sequence Variants (ASVs) count comparison among four samples (MS, FS, WS, SS) based on the most abundant and common genera. The results indicate that the most abundant genera across all samples were *Vibrio*, particularly notable in female *H. kuda* samples, except in soil. The genus *Vibrio*, classified under the phylum Proteobacteria and the family Vibrionaceae, comprises ubiquitous bacteria found in various marine and aquatic environments, known to cause infections in both human and aquatic animals (Baker-Austin et al. 2018; Sampaio et al. 2022). Several reports have documented *Vibrio* species causing diseases in seahorses and other marine invertebrates, including *V. harveyi*, *V. vulnificus* (Binh et al. 2016), *V. alginolyticus*, and *V. splendidus* (Balcázar et al. 2010).

The genus *Roseobacter*, in the family Rhodobacteraceae in the phylum Proteobacteria, stands out as one of the nine most prevalent bacterial lineages across marine environments. It demonstrates considerable abundance in the pelagic zone and biofilms associated with algae (Haggerty and Dinsdale 2017; Seo et al. 2017). *Roseobacter* primarily contributes to carbon and sulfur biochemical cycling and establishes symbiotic relationships with various aquatic macro and micro-organisms (Pujalte et al. 2014). Whereas, *Candidatus*, a genus within the family Actinomarinaceae, commonly referred to as OM1, plays a pertinent role in the carbon cycle of the ocean (López-Pérez et al. 2020; Ulloa et al. 2021; Henson and Thrash 2023).

The genus *Photobacterium*, a member of the Vibrionaceae family within the phylum Proteobacteria, comprises Gram-negative, motile, facultative bacteria commonly found in association with marine organisms (Lo et al. 2014; Li et al. 2017; Wang et al. 2017). These bacteria occur as free-living organisms in seawater and sediments and in symbiotic relationships with marine animals, including the bioluminescent strains found within the light organs of deep-sea fish (Labella et al. 2017). Certain species of Photobacterium, such as *P. phosphoreum* and *P. iliopiscarium*, are known for spoiling chilled fish and seafood products in the food industry (Fuertes-Perez et al. 2019). *Photobacterium iliopiscarium* species have been reported in various studies to be associated with and isolated from marine fishes (Thyssen and Ollevier 2015; Hilgarth et al. 2018).

The genus *Flavobacterium*, classified within the Flavobacteriaceae family under the phylum Bacteroidetes, is Gram-negative and is commonly referred to as the "Cytophaga-Flavobacterium-Bacteroides group," which currently encompasses more than 50 genera (Waśkiewicz and Irzykowska 2014; Chong 2022). *Flavobacterium* species are abundant in both freshwater and marine environments, where they play a specialized role in the uptake, degradation, and decomposition of organic matter in aquatic ecosystems, as well as in bacterioplankton biomass. Notably, these species can hydrolyze organic polymers (Waśkiewicz and Irzykowska 2014). Several species within the genus are recognized as causative agents of diseases, such as *F. columnare*, responsible for columnaris disease in freshwater fish (LaFrentz et al. 2014), and *F. psychrophilum*, which causes peduncle disease in rainbow trout (Chong 2022).

The genus *Pseudoalteromonas*, part of the family Pseudoalteromonadaceae, is widely distributed throughout the marine environment, particularly in deep-sea and polar regions. It is associated with a diverse array of marine organisms, such as fishes, sponges, mollusks, and corals, as well as sediments and seawater (Liu et al. 2019; Wei et al. 2021; Zheng et al. 2023). Members of the genus *Pseudoalteromonas* are known to produce various bioactive compounds possessing antibacterial, antifungal, antifouling, and algicidal properties (Zeng et al. 2015; Atencio et al. 2018).

The genus *Ruegeria*, classified within the family Rhodobacteraceae in the class Alphaproteobacteria, is commonly isolated from marine environments, aquatic bacteria, and marine invertebrates. It primarily participates in carbon and sulfur biogeochemical cycling and establishes symbiotic relationships with aquatic macro- and micro-organisms (Kämpfer et al. 2013; Pujalte et al. 2014).

The genus *Synechococcus*, belonging to the family Synechococcaceae in the phylum Cyanobacteria, plays a significant role in carbon fixation within aquatic habitats. Members of this genus are among the most abundant photo-oxygenic micro-organisms involved in carbon fixation and are predominantly found in marine environments, particularly in tropical freshwater. They exist either in free-living states or in symbiotic relationships with animals and plants (Dvořák et al. 2014; Komárek et al. 2020).

The genus *Altererythrobacter*, a member of the family Erythrobacteraceae in the Alphaproteobacteria class, comprises Gram-negative bacteria with epoxide hydrolase activity. Members of this genus are commonly isolated from a diverse range of environments, including marine, aquatic, and terrestrial habitats, as well as from various organisms (Yang et al. 2014; Kang et al. 2016; Xue et al. 2016; Fidalgo et al. 2017; Yan et al. 2017; Yuan et al. 2017; Dahal and Kim 2018; Xue et al. 2021).

Functional prediction of microbiome functional composition

Moreover, the prediction of microbiome function composition in both skin samples and environmental samples at the KEGG pathway level 2 was conducted using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis. The results revealed functional predictions associated with various metabolic pathways, including carbohydrate metabolism, amino acid metabolism, protein metabolism, energy metabolism, nucleotide metabolism, lipid metabolism, cofactors and vitamins metabolism, and glycan biosynthesis and metabolism. Predictions were made for genetic information processing pathways such as translation, replication and repair, and folding sorting and degradation. Environmental information processing pathways, including membrane transport, signal transduction, and cellular processing pathways like cellular community prokaryote and cell motility, were also identified (Figure 5). Functional prediction offers an initial overview of the bacterial communities thriving in the skin of both male and female *H. kuda and* their surrounding environment (water and soil/sediment). These communities contribute to seahorse survival and therapeutic properties, while also serving as a potentially valuable source of cellulolytic enzymes that can be further utilized for various applications such as biofuel production, biotechnology, and bioremediation processes.

Bacteria possess various metabolic pathways, enabling them to utilize diverse nutrients. These nutrients are vital for the development, maintenance, and function of the host immune system, as well as overall health (Epstein et al. 2019; Alsaffar et al. 2020; Marchioro et al. 2020; Risely 2020; Wainwright et al. 2020; Costantini et al. 2021).

The bacterial metabolism of carbohydrates in seahorses has significant implications for the seahorses and their surrounding environment. Bacteria utilize a variety of carbohydrates, including glucose, sucrose, and fructose, as energy sources (Jeckelmann and Erni 2020). Certain bacteria can degrade complex carbohydrates like starch and cellulose, commonly found in fish feed and the environment (Hua et al. 2022). Carbohydrate metabolism in the skin contributes to processes such as the degradation of organic matter in aquatic environments (Zinke et al. 2019), energy production for bacterial growth, and the generation of metabolic byproducts that influence the surrounding environment (Passalacqua et al. 2016). For example, bacterial metabolism of carbohydrates can synthesize organic acids such as acetic acid, propionic acid, and lactic acid, which can lower the pH of the surrounding water (Liu et al. 2020; Qiao et al. 2020). As a result, bacterial growth and the byproducts of carbohydrate metabolism, which drive energy supply, may impact the development and survival of seahorses and other aquatic animals.

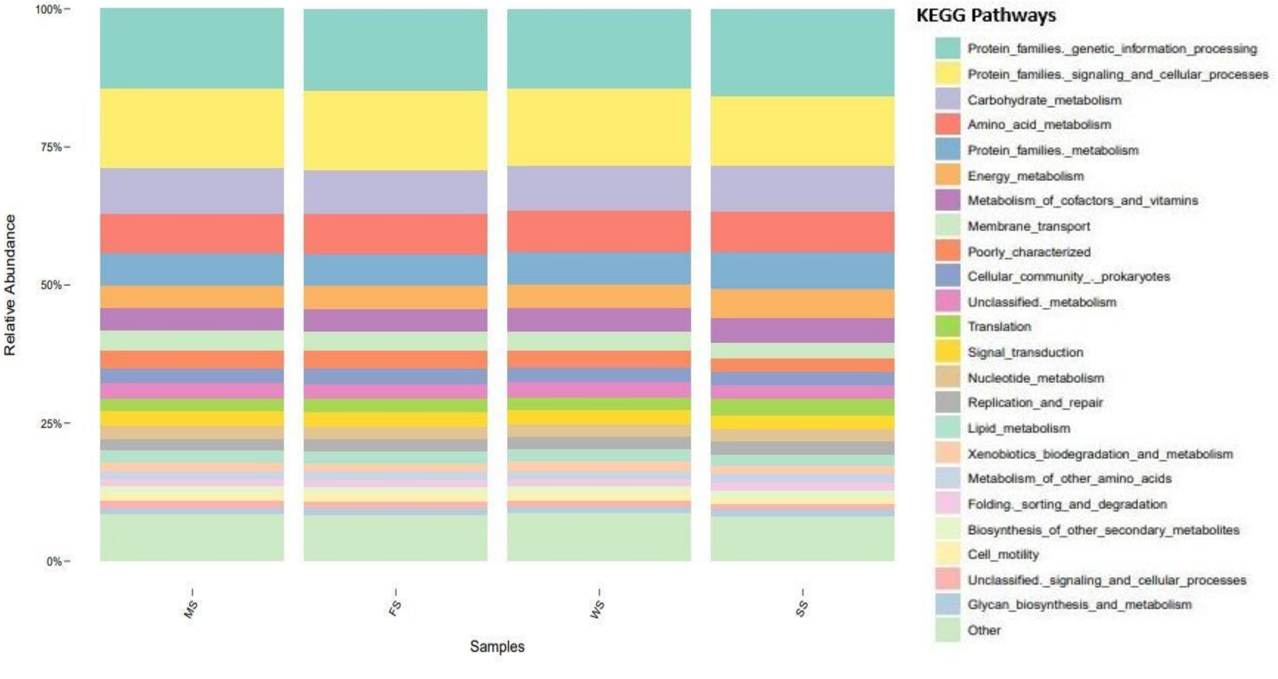

Figure 5. PICRUSt analysis of predictive functional analysis of bacterial communities as represented by KEGG pathways for four samples (epithelial surface of the male and female seahorses, water, and soil/sediment)

Carbohydrates and energy metabolism are closely linked, with carbohydrates being the primary energy source. Bacterial cells metabolize carbohydrates via glycolysis to produce energy, breaking down glucose to generate ATP. This process occurs aerobically in the skin or anaerobically in the gut, depending on oxygen availability (Puccio et al. 2021). The carbohydrate and energy metabolism of the microbiome in seahorses can significantly influence their health and performance. For instance, the energy metabolism of bacteria observed in assessments of the human gut microbiome has been found to impact the development and pathology of the endocrine system (Qi et al. 2021). Consequently, the fundamental principles of carbohydrate and energy metabolism in bacteria may affect body composition, growth rate, and reproductive performance, all of which are influenced by the microbiome in the skin and gut of seahorses. The carbohydrate and energy metabolism of bacteria can affect the production of metabolic byproducts, thereby altering the pH of surrounding waters (Wang et al. 2022), which could also impact the growth and survival of seahorses.

Meanwhile, amino acids are essential biomolecules that serve as the building blocks of proteins and intermediates in various metabolic pathways (Mohanty et al. 2014). In animal cells, amino acids play important roles in cell signaling and act as regulators of gene expression and protein phosphorylation cascades (Wu 2010), as well as in nutrient transport and metabolism (Wang et al. 2013), and innate and cell-mediated immune responses. These observations suggest that functional annotations related to amino acid metabolism in the skin of seahorses may contribute to processes such as amino acid synthesis, degradation, and recycling. The metabolism of cofactors and vitamins has physiological effects on host immunity and other biological responses (Yoshii et al. 2019). Bacteria in the skin of seahorses can affect their immunity.

Nucleotide metabolism is vital for bacterial survival, supplying the essential components needed for DNA and RNA synthesis (Zhao et al. 2021). Additionally, it plays a role in antibiotic-induced mortality in bacterial pathogens, potentially leading to treatment failure if abnormalities occur (Lopatkin and Yang 2021). While research on nucleotide metabolism in seahorses is scarce, the observed bacterial nucleotide metabolism in seahorse skin microbiomes could aid in their survival by producing compounds that inhibit the colonization and growth of pathogenic bacteria (Goncheva et al. 2022).

Functional annotations associated with lipid metabolism in seahorse skin microbiota suggest the potential participation of both skin and gut microbiomes in lipid breakdown, modification, and utilization. Lipids play essential roles in diverse biological functions, such as energy storage (Parzanini et al. 2018). Additionally, they are crucial components of cell membranes and contribute to various cellular and physiological processes essential for organisms' overall survival, growth, and reproductive success (Bergé and Barnathan 2005; Glencross 2009; Parzanini et al. 2018).

Functional annotation related to glycan biosynthesis and metabolism was identified. Bacteria engage in synthesizing and metabolizing glycans for energy or as carbon sources, exemplified by the breakdown of chitin by marine bacteria. This process involves the secretion of chitinases, which cleave glycosidic bonds between monosaccharides (Koropatkin et al. 2012; Rathore and Gupta 2015). The resulting oligosaccharides are then absorbed by bacteria for energy metabolism (Rowland et al. 2018). Furthermore, glycan biosynthesis and metabolism may utilize glycans as surface decorations or virulence factors in pathogenic organisms (Varki 2017; Williams et al. 2020). Consequently, bacteria involved in this metabolic pathway may contribute to the host's defense mechanism, potentially safeguarding the dermal epithelium of *H. kuda* during pathogen attacks and invasions.

Another metabolic pathway identified is genetic information processing, specifically translation. In this process, bacteria produce proteins that contribute to various functions, including adhesion to the epithelial surface, competition for resources with other micro-organisms, aiding in digestion, and providing essential nutrients (Patti et al. 1994; Zheng et al. 2021). These proteins are then released and distributed in the skin, assisting commensal bacteria in adherence and promoting a healthy microflora. This process also aids in the complete digestion of the remnants consumed by seahorses, potentially influencing their overall physiological responses.

In environmental metabolism processes, membrane transport and signal transduction pathways were identified. Bacteria inhabiting the skin microbiome rely on efficient membrane transport mechanisms to acquire nutrients and essential molecules for survival. Various processes facilitate membrane transport: Gases and water are transferred through passive diffusion, while specific amino acids and carbohydrates are transported through channels or transporters via facilitated diffusion (Cooper 2000). Outer Membrane Vesicles (OMVs) play a crucial role in transporting molecules across membranes and distributing them to other bacterial cells or the external environment (Kulp and Kuehn 2010; Furuyama and Sircili 2021; Magaña et al. 2023). Consequently, bacteria can uptake nutrients and other molecules from the environment, potentially influencing the physiological immune response of seahorses over time.

Additionally, signal transduction is a vital mechanism for bacteria to interpret environmental cues and adjust their responses accordingly (Jung et al. 2018; Bridges et al. 2022). One prevalent mechanism in bacterial signal transduction involves two-component signaling, where a membrane-bound sensor protein detects environmental signals, and a cytoplasmic response regulator modulates gene expression or other cellular processes in response (Mitrophanov and Groisman 2008; Zschiedrich et al. 2016). This mechanism enables bacteria to sense changes in nutrient availability or oxidative stress and activate specific gene expression programs to adapt accordingly. Moreover, bacteria employ diverse sensory systems to detect various environmental cues, including nutrients, toxins, temperature, pH, and light, converting this information into intracellular signals that prompt appropriate responses (Clausznitzer et al. 2014; Jung et al. 2018). Therefore, bacteria associated with seahorses potentially aid in the uptake of nutrients and the release of antioxidant enzymes to counteract reactive oxygen stress and safeguard seahorse epithelial integrity.

Moreover, certain skin bacteria can breakdown and metabolize external substances like pollutants or toxins. Through xenobiotic metabolism, they detoxify harmful foreign chemicals, while secondary metabolism enables them to combat competitors and thrive in challenging chemical environments chemically (Kontomina et al. 2022). The functional annotations associated with xenobiotic biodegradation and metabolism suggest the potential involvement of the *H. kuda* skin microbiome in detoxification processes, resilience against environmental stressors, and adaptation to exposure to foreign substances.

In conclusion, the most frequently observed (ASVs) were associated with the genera *Vibrio*, *Roseobacter*, *Photobacterium, Ruegeria, Candidatus, Pseudoalteromonas*, *Synechococcus*, *Flavobacterium*, and *Altererythrobacter* and some unidentified bacteria, which suggest normal microflora of seahorse that may interact to the host either beneficial or pathogenic. Moreover, the KEGG pathway revealed microbiome function associated with the skin of seahorses mainly on annotations such as metabolism, genetic information processing, environmental information processing, and cellular processing. These functions help us understand how the bacteria community affects the growth and health of *H. kuda* and highlight the central role of the skin microbiota in various metabolic and cellular processes. Thus, this study gives valuable insights into the taxonomic composition of the bacterial community associated with *H. kuda* and its surrounding environment, as well as functional annotation that may shed light on the metabolic adaptability, and dynamics of the microbiome associated with seahorse that can help in achieving the long term goal of alleviating, protecting, and producing the population of seahorses.

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