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STUDY OF ENZYMATIC AND ANTIBACTERIAL ACTIVITIES OF HORSESHOE CRAB GUT SYMBIONTS AND AMINO ACID CONTENT OF EGGS AS SUPPORTING PHYSIOLOGICAL ASPECTS

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ABSTRACT

Horseshoe crabs (*Tachypleus gigas*) inhabit dynamic coastal ecosystems, where gut-associated symbiotic bacteria play essential roles in host physiology and represent a promising source of marine bioactive compounds. This study investigated the enzymatic and antibacterial activities of gut symbionts isolated from *T. gigas* and examined their potential functional association with the amino acid composition of the eggs as a supporting physiological aspect. Gut bacteria were isolated using Marine Agar and characterized based on colony morphology, hydrolytic enzyme production (amylase, lipase, protease, and cellulase), and antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. The most bioactive isolates were identified through 16S rRNA gene sequencing, while amino acid profiling of the eggs was conducted to assess their nutritional and bioactive potential. The results revealed high morphological and functional diversity among gut symbionts. Isolates H.A.1.2 and H.A.1.5 exhibited broad-spectrum antibacterial activity against both Gram-negative and Gram-positive bacteria. Distinct enzymatic activities were observed, including amylase production by H.B.2.3, lipase by H.C.4.3, cellulase by H.A.1.5, and protease by H.A.1.2, indicating their roles in macromolecule degradation and nutrient recycling. Amino acid analysis showed a total amino acid content of 59.32%, dominated by essential amino acids such as leucine (5.72%), isoleucine (4.72%), lysine (3.98%), and valine (3.97%), as well as non-essential amino acids, including glutamate (7.91%) and aspartate (7.27%). The enzymatic activity of gut symbionts is likely linked to the availability of free amino acids necessary for embryonic development and vitellogenesis. Molecular identification revealed that H.A.1.2 was closely related to *Pseudoalteromonas piscicida*, while H.A.1.5 showed close similarity to *Vibrio alginolyticus*. Overall, this study highlights the functional interplay between gut symbiotic bacteria and egg biochemical composition in *T. gigas*, underscoring its potential as a sustainable source of antibacterial agents, industrial enzymes, and marine-derived bioactive metabolites.

Keywords: *Tachypleus gigas,* Gut symbionts, Bioactive compounds, Enzymatic-Antibacterial activity.

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1. INTRODUCTION

Coastal and marine ecosystems represent reservoirs of exceptionally high biodiversity and hold tremendous potential for the development of modern biotechnology. Benthic organisms and marine invertebrates, which generally possess long evolutionary lineages, exhibit unique physiological adaptations and maintain close ecological relationships with symbiotic microbial communities. Scientific interest in these organisms extends beyond taxonomy and conservation to include their potential as sources of secondary metabolites, enzymes, and bioactive compounds with high value for industrial, pharmaceutical, and environmental applications (Imhoff, 2016). In this context, the marine environment serves as a strategic genetic resource for sustainable bioprospecting.

One of the primary focuses in marine biotechnology is the exploration of associated microorganisms, particularly symbiotic microbes residing in host digestive tracts. The gut microbiota plays essential roles in digestion, nutrient absorption, and host physiological adaptation to dynamic environmental conditions. In addition, symbiotic microorganisms produce a wide range of bioactive metabolites that function as chemical defense mechanisms against pathogens (Kumari et al., 2021). Numerous studies have demonstrated that bioactive compounds previously attributed to marine animal hosts are, in fact, synthesized by their internal

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microbial symbionts, positioning exploration of the gut microbiota as a key approach in marine biotechnology development (Imhoff, 2016).

The horseshoe crab (*Tachypleus gigas*) represents a particularly compelling marine invertebrate for investigating host–symbiont interactions. Often referred to as a "living fossil," this organism has maintained a relatively unchanged morphology for more than 500 million years of evolution (Jia et al., 2023). Such evolutionary resilience reflects a complex and stable physiological system, which is hypothesized to be supported, at least in part, by symbiotic microbial communities. Beyond its ecological importance in coastal ecosystems, the T. gigas holds significant biotechnological value, notably through the utilization of hemolymph containing hemocyanin in the development of Limulus Amebocyte Lysate (LAL) and Tachypleus Amebocyte Lysate (TAL) for endotoxin detection (Guryanova et al., 2023). However, compared with extensive studies on hemolymph, research on the gut microbiota of *T. gigas* remains limited, particularly regarding the functional roles of symbiotic bacteria.

Investigations of the gut microbiota of *T. gigas* offer strategic advantages, as they are minimally invasive and enable the discovery of novel bioactive resources without overexploiting natural populations. Gut microbial communities are known to comprise several dominant phyla, including Bacteroidetes, Proteobacteria, Tenericutes, Firmicutes, and Fusobacteria, which contribute to host metabolism, digestion, and immune function (Wang et al., 2020). Certain symbiotic bacteria have been reported to produce hydrolytic enzymes such as amylase, lipase, cellulase, and protease (Blair et al., 2021), as well as antibacterial compounds that protect the host from pathogenic microorganisms (Alexpandi et al., 2019). These enzymatic and antibacterial activities are not only crucial for host physiology but also possess substantial applied value in the development of environmentally friendly biotechnological products.

In addition to their roles in enzyme production and bioactive metabolite synthesis, gut microbiota also contribute to amino acid metabolism and availability within the host. Symbiotic microbes are involved in the biosynthesis, degradation, and transformation of amino acids, thereby influencing the nutritional balance and physiological condition of the host (Lin et al., 2017; Han et al., 2025). In the context of reproduction, the amino acid composition of eggs can reflect the host's metabolic and physiological status and may be indirectly influenced by the functional activities of the gut microbiota. Nevertheless, studies linking the functional activity of gut symbionts with egg amino acid composition in *T. gigas* remain extremely limited. An integrated research approach combining the characterization of gut symbiotic bacteria based on enzymatic and antibacterial activities with analysis of egg amino acid composition is expected to provide a more comprehensive understanding of the functional relationship between symbionts and their host. Therefore, this study aimed to identify gut symbiotic bacteria associated with *T. gigas*, evaluate their enzymatic and antibacterial activities, and analyze egg amino acid content as a supporting physiological parameter. This research aligns with the concept of blue biotechnology, which emphasizes the responsible utilization of marine biodiversity to generate sustainable biotechnological knowledge and innovation (Maldonado-Ruiz et al., 2024).

Based on these considerations, the objectives of this study were to identify gut symbiotic bacteria of *T. gigas* and to evaluate their enzymatic activity and antibacterial potential. Additionally, proximate analysis and egg amino acid profiling were conducted to assess host nutritional status. The integration of these approaches was intended to elucidate the functional role of symbiotic bacteria in supporting metabolism and reproduction in *T. gigas*.

2. MATERIALS AND METHODS

2.1. Sample Collection and Identification

T. gigas specimens were collected from the coastal waters of Tugu District, Semarang City, Indonesia. *In situ* measurements of environmental parameters, including temperature, pH, and salinity, were recorded at the sampling site. Individual *T. gigas* were placed in sterile, labeled containers and immediately transported to the laboratory for further analysis. Species identification was conducted based on external morphological characteristics, with reference to the World Register of Marine Species (WoRMS) database.

2.2. Bacterial Isolation

Samples were collected from the dorsal region, extending from the foot area to the upper body. Symbiotic bacteria were isolated from intestines that were aseptically removed and homogenized in a mortar and pestle with sterile seawater added. One milligram of the homogenized intestinal sample was transferred into a Falcon tube and serially diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. Each dilution was spread onto Petri dishes containing Nutrient Agar (NA) supplemented with nystatin. The plates were sealed with plastic film and incubated at 28°C for 5–7days.

2.3. Macroscopic Purification and Identification of Bacteria

Purification was carried out by selecting a single bacterial colony with a sterile inoculating loop and transferring it to fresh NA medium prepared with seawater. Isolates obtained from the initial isolation were further

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purified using the four-quadrant streak method to obtain pure cultures. The inoculated plates were sealed, labeled, and incubated at room temperature (28°C) for 1–3days. Purified symbiotic bacterial isolates were subsequently characterized macroscopically based on colony morphology, including shape, margin, size, color, and elevation (Tarigan et al., 2023).

2.4. Enzymatic Activity Test

Enzymatic activity assays were performed on all symbiotic bacterial isolates to assess amylase, protease, cellulase, and lipase activities. Each isolate was first cultured in broth medium for 24 hours with shaking at 110rpm at room temperature. Enzymatic assays were conducted following the method described by Setyati et al. (2023). Enzyme-specific media were prepared as follows: 1) amylase activity on agar supplemented with 1% soluble starch; 2) protease activity on agar supplemented with 1% skim milk; 3) cellulase activity on agar supplemented with 1% carboxymethyl cellulose (CMC); and 4) lipase activity on agar supplemented with 1% Tween-80. All plates were incubated at 28°C for 24hours.

2.5. Screening of Antibacterial Activity

All symbiotic bacterial isolates were screened for antibacterial activity using the agar plug method. The pathogenic test strains used were *Escherichia coli* and *Staphylococcus aureus*. The screening procedure followed the method described by Sabdono et al. (2022). Test pathogens were refreshed on Nutrient Agar (NA) and incubated at 37°C for 24 hours. The cultures were then transferred to Nutrient Broth (NB) and incubated with shaking at 100rpm at room temperature for 24hours. The turbidity of the pathogen suspensions was adjusted to a 0.5 McFarland standard. Mueller–Hinton Agar (MHA) plates were evenly swabbed with the standardized pathogen cultures using sterile cotton swabs. Inhibition zones formed around the agar plugs were measured using a vernier caliper (Wijaya et al., 2022).

2.6. Production and Extraction of Bacterial Metabolites

Bacterial isolates exhibiting antibacterial activity were precultured in 50mL of Nutrient Broth (NB) for 3 × 24 hours with shaking at 28°C and 110rpm. The pre-cultures were subsequently transferred into 250mL of fresh culture medium and incubated with shaking at 28°C and 110rpm for 6 days. Metabolite extraction was carried out using liquid–liquid extraction with ethyl acetate at a 1:1 (v/v) ratio, followed by homogenization for 24hours. The mixture was centrifuged at 5000rpm for 10minutes, and the resulting supernatant was concentrated using a rotary evaporator to obtain a dry crude extract.

2.7. Bacterial Extract Activity Test

The antibacterial activity of the crude extracts was evaluated using the agar diffusion method. The dried extracts were prepared at a concentration of 1000 µg/disc and applied to 6mm sterile paper discs. *E. coli* and *S. aureus* were cultured in Nutrient Broth (NB) and incubated with shaking at 110rpm and 28°C for 24hours. Bacterial turbidity was adjusted to a 0.5 McFarland standard, and the suspension was evenly swabbed onto Mueller–Hinton Agar (MHA) plates using sterile cotton swabs. Extract-impregnated discs were placed on the inoculated MHA surface using sterile tweezers. The extraction solvent served as the negative control, and chloramphenicol as the positive control. All assays were conducted in duplicate, and inhibition zones were measured using a caliper.

2.8. Molecular Identification of Bacterial Isolates

Molecular identification of the two symbiotic bacterial isolates exhibiting the strongest antibacterial activity was performed using 16S rRNA gene sequencing (Pringgenies et al., 2024). The amplified gene fragments were compared with reference sequences in the NCBI database using the Basic Local Alignment Search Tool (BLAST) to determine their closest taxonomic affiliations.

2.9. Antibacterial Activity of *T. gigas* Body Extracts

Body extract preparation was conducted using the maceration method with 96% ethanol (Aisa & Tukiran, 2023). *T. gigas* body material was thoroughly cleaned, oven-dried for 48hours, and ground into a fine powder. Approximately 12–15g of the powder was macerated in ethanol at a 1:10 (w/v) ratio and shaken at 150rpm for 48 hours. The macerate was filtered, and the filtrate was concentrated using a rotary evaporator at 40°C and 70rpm until a viscous extract was obtained. The extract was stored under refrigerated conditions. Antibacterial activity was evaluated against *S. aureus* and *E. coli* using the disk diffusion method. The extract was dissolved in dimethyl sulfoxide (DMSO) at concentrations of 250, 500, and 1000 µg/disc and applied to sterile paper discs. The discs were placed on MHA plates inoculated with bacterial suspensions standardized to 0.5 McFarland. Plates were incubated at 28°C for 72 hours, and observations were recorded at 24-hour intervals. Inhibition zone diameters were measured using a caliper.

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2.10. Proximate Analysis of Eggs

Proximate analysis of *T. gigas* eggs was performed to determine moisture, ash, fat, protein, and carbohydrate contents. Moisture content was determined following AOAC (2005), as cited in Salsabila et al. (2024), by drying 2–3g of sample at 105°C until a constant bwt was achieved. Ash content was measured according to Tjalo et al. (2024) by incinerating 1–2g of sample in a furnace at 600°C for 4hours. Fat content was analyzed using the Soxhlet extraction method with *n*-hexane as the solvent at 60°C for 5hours (Ishak et al., 2023). Protein content was determined using the Bradford method (Rekowski et al., 2021), with modifications from Wilder & Barnes (2023). Briefly, 5–9mg of the sample was dissolved in 0.1 M NaOH, incubated at 80°C for 30minutes, centrifuged, and the resulting supernatant was reacted with Bradford reagent. Absorbance was measured at 595nm using a spectrophotometer. Carbohydrate content was determined using the DNS method (Namboonlue et al., 2025), employing a glucose standard solution (0–100mg/mL) and measuring absorbance at 540nm. All analyses were performed in duplicate, and results were expressed as percentages (% dry bwt).

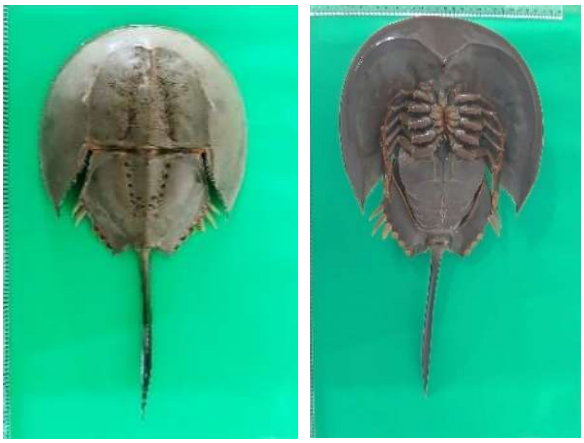
2.11. Amino Acid Profile Analysis

Amino acid profiling was performed using High-Performance Liquid Chromatography (HPLC) following acid hydrolysis with 6M HCl at 100°C for 24hours. Separation was performed on an AccQ C18 column with fluorescence detection. Amino acids were identified by comparing sample retention times with those of amino acid standards (Ishak et al., 2023).

3. RESULTS AND DISCUSSION

3.1. Identification of *T. gigas* Specimens

Based on morphological identification (Fig. 1), the horseshoe crab specimens collected from the study site were classified as *T. gigas* (Müller, 1785). Identification followed the diagnostic morphological features described by Fauziyah et al. (2023).



A B

Fig. 1: Morphological appearance of Horseshoe crabs (*Tachypleus gigas*). A) Dorsal view, and B) Ventral view.

Overall, the specimens exhibited the typical characteristics of the genus *Tachypleus*, including a horseshoe-shaped body with a semicircular prosoma (anterior carapace) and a pair of large compound eyes positioned laterally. The examined individual measured 43.5cm in total length, with a prosoma width of 24.8cm and a telson length of 18.5cm. Body coloration was predominantly grayish-brown, and the carapace was hard, smooth, and glossy. The opisthosoma possessed six pairs of robust, tapered marginal (lateral) spines, which represent one of the distinguishing traits of this species. The telson was long, slender, and pointed, with a triangular cross-section, consistent with the typical morphology of *T. gigas*, a species commonly inhabiting sandy to muddy coastal substrates.

The *T. gigas* specimens from the waters of Tugu District, Semarang City, were comparatively larger than those reported from other regions, such as Tanjung Punai Beach, West Bangka (Fuad et al., 2024), the coastal waters of Balikpapan (Nuraisah et al., 2020), and the estuarine waters of South Sumatra (Fauziyah et al., 2021). This larger morphometric size may be associated with favorable environmental conditions that support growth, as well as the advanced age of individuals that have reached full maturity. According to Sekiguchi et al. (1988), adult *T. gigas* typically exhibit increased prosoma width after their 13th molt. Thus, regional variation in morphometric size reflects the combined influence of biological factors and local environmental conditions on growth patterns.

The examined specimen was identified as female, based on its relatively large body size, reduced terminal marginal spine, two-segmented operculum, and slender pedipalps resembling walking legs. In contrast, males are generally smaller and possess modified chelae on the first pair of legs, which function to grasp females during copulation. These observations are consistent with previous reports (Maulana et al., 2023; Fuad et al., 2024), which indicate that female *T. gigas* are typically larger than males due to differences in hormonal regulation and molting patterns. Furthermore, the hook-like structure of male pedipalps serves a reproductive function, whereas females

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lack this modification. Sampling was conducted in a muddy intertidal zone adjacent to a mangrove area, characterized by a soft substrate and relatively high salinity levels (25–30%). These conditions are consistent with the preferred natural habitat of *T. gigas* as illustrated in Fig. 1.

3.2. Morphology of Gut Symbiotic Bacterial Colonies of *T. gigas*

Intestinal symbionts in marine animals play a crucial role in host physiology, including digestion, metabolism, and biological defense. In *T. gigas*, the intestinal microbial community isolated in this study exhibited high morphological and physiological diversity, suggesting a complex and functionally important symbiotic relationship between the host and its associated microorganisms.

Isolation of bacterial symbionts from the digestive tract revealed several colonies with distinct morphological characteristics (Fig. 2). Colonies grown on Marine Nutrient Agar (MNA) at room temperature showed variation in form, pigmentation, elevation, and margin structure (Table 1).

Table 1: Morphological Identification of Symbiotic Bacterial Isolates

Isolat	Morphology				
	Shape	Color	Margin	Elevation	Size
H.C.4.3	Circular	Yellow	Entire	Convex	Small
HA.4.1	Circular	White	Entire	Flat	Small
H.A.1.2	Circular	Yellow	Undulate	Flat	Small
H.B.2.3	Circular	White	Undulate	Flat	Small
H.A.2.2	Irregular	White	Entire	Flat	Small
H.A.1.5	Circular	White	Serrate	Flat	Small
H.B.2.1	Circular	White	Undulate	Flat	Small
H.B.4.2	Irregular	Cream	Undulate	Flat	Small
H.A.3.2	Circular	Cream	Entire	Flat	Punciform

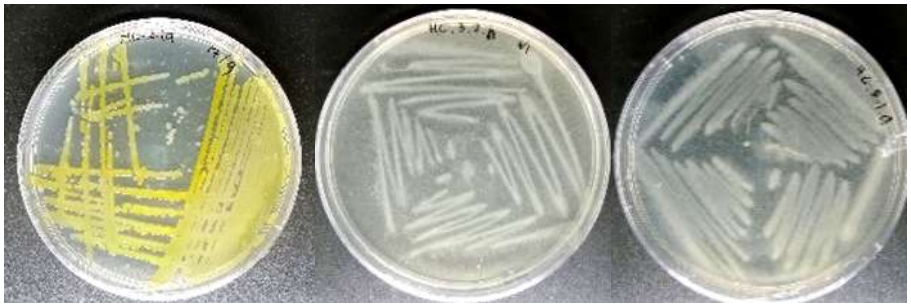

Fig. 2: Morphology of symbiotic bacterial colonies.

Overall, differences in colony color and morphology indicate substantial diversity among the gut symbionts of *T. gigas*, likely reflecting variation at the genus level or differences in metabolic capabilities among isolates. These morphological traits provide an essential foundation for subsequent analyses, including Gram staining, biochemical assays, and antibacterial activity testing, which collectively help elucidate each symbiont's biological potential. The predominance of yellow and white colonies may indicate the ability to biosynthesize secondary metabolites, a trait of particular interest for the discovery of novel bioactive compounds from marine symbionts. Ecologically, the roles of these microorganisms extend beyond decomposing complex organic matter. They also contribute to the synthesis of essential amino acids and bioactive compounds that support gut microbiota homeostasis. According to Han et al. (2025), the gut microbiota of marine organisms can regulate amino acid availability and metabolism through complementary biosynthetic and degradative pathways. In the context of *T. gigas*, these symbionts likely enhance metabolic efficiency and physiological adaptation in dynamic, organic-rich coastal sediment environments.

3.3. Enzymatic Activity Assay of *T. gigas* Gut Symbionts

Enzymatic activity assays for amylase (amylolytic), lipase (lipolytic), cellulase (cellulolytic), and protease (proteolytic), expressed as Enzyme Activity Index (mm), are presented in Table 2.

Tests conducted on nine bacterial isolates revealed considerable variation in their capacity to produce hydrolytic enzymes. All isolates showed varying levels of activity across enzyme types (Table 3). The highest amylolytic activity was observed in isolate H.B.2.3 (2.64mm), followed by H.B.2.1 (2.63mm) and H.A.3.2 (2.50mm). Several isolates, including H.A.4.1 and H.A.1.5, showed no detectable amylolytic activity (0.00mm). Lipolytic activity was highest in isolates H.C.4.3 (2.65mm) and H.A.2.2 (2.35mm), whereas the lowest activity was recorded in H.B.4.2 (1.38mm) and H.B.2.1 (1.50mm).

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Table 2: Enzymatic Index of Horseshoe Crab Gut Symbionts

	Enzyme Activity Index (mm)			
Isolate Code	Amylolytic	Lipolytic	Cellulolitic	Proteolytic
H.A.1.2	1.59	2.07	1.54	2.55
H.A.2.2	2.34	2.35	1.82	0.25
H.B.2.1	2.63	1.50	2.15	0.00
H.A.3.2	2.50	1.55	2.26	0.00
H.B.2.3	2.64	2.21	1.73	1.66
H.A.4.1	0.00	0.00	1.95	1.45
H.B.4.2	2.49	1.38	1.82	0.00
H.C.4.3	2.33	2.65	1.60	0.00
H.A.1.5	0.00	0.00	2.42	0.00

Table 3: Antibacterial Screening of Horseshoe Crab Gut Symbionts

Isolate code	*E.coli*				*S.aureus*			
	24hour		48hour		24hour		48hour	
	1	2	1	2	1	2	1	2
H.A.1.2	+	+	+	+	+	+	+	-
H.A.2.2	-	-	-	-	-	-	-	-
H.B.2.1	-	-	-	-	-	-	-	-
H.A.3.2	-	-	-	-	-	-	-	-
H.B.2.3	+	+	+	+	-	-	-	-
H.A.4.1	-	-	-	-	-	-	-	-
H.B.4.2	-	-	-	-	-	-	-	-
H.C.4.3	-	-	-	-	-	-	-	-
H.A.1.5	+	+	+	+	-	+	-	+

In cellulolytic assays, most isolates exhibited activity index values ranging from 1.54 to 2.42mm, with the highest values recorded in H.A.1.5 (2.42mm) and H.A.3.2 (2.26mm). Proteolytic activity varied substantially among isolates, with the highest activity detected in H.A.1.2 (2.55mm) and H.B.2.3 (1.66mm). Several isolates, including H.B.2.1, H.A.3.2, H.B.4.2, H.C.4.3, and H.A.1.5, showed no detectable proteolytic activity. These variations in enzyme activity indices reflect differences in the physiological potential of gut symbionts to degrade complex organic compounds within the digestive environment of *T. gigas*.

Microbial hydrolytic activity contributes to the breakdown of carbohydrates, lipids, proteins, and fibrous organic materials derived from ingested substrates. In the digestive tract of *T. gigas*, enzymes play crucial roles in metabolism, digestion, and biomolecule synthesis (Li et al., 2023). Amylase facilitates the hydrolysis of starch into simple sugars such as glucose and maltose through the cleavage of α-glycosidic bonds (Jujjavarapu & Dhagat, 2019; Samanta, 2022), thereby contributing to the energy availability required to sustain energetically demanding reproductive processes. Proteases further support this process by hydrolyzing proteins into short peptides and free amino acids, which are essential for physiological functions (Kumari et al., 2015; Singh et al., 2019). In *T. gigas*, amino acids released through bacterial protease activity are hypothesized to act as key precursors for yolk protein (vitellin) synthesis.

In addition, symbiotic bacteria produce lipases that hydrolyze triglycerides into fatty acids and glycerol at the lipid–water interface (Filho et al., 2019). These lipid derivatives represent high-energy reserves stored in eggs to support embryonic development (Chandra et al., 2020). The presence of cellulolytic enzymes further suggests an adaptive advantage, enabling *T. gigas* to exploit plant-derived detritus in mangrove ecosystems. Cellulases hydrolyze β-1,4-glycosidic bonds in cellulose through the synergistic action of endoglucanases, exoglucanases, and β-glucosidases, ultimately releasing glucose as an additional energy source (Sharma et al., 2016; Jayasekara & Ratnayake, 2019).

The findings of this study are consistent with previous reports (Li et al., 2016; Shindoh et al., 2021), demonstrating that bacterial genera such as *Vibrio*, *Photobacterium*, and *Pseudoalteromonas* can produce extracellular proteases and aminopeptidases that efficiently degrade natural protein substrates. Physiologically, the enzymatic activities of these symbionts support ecological adaptation in *T. gigas* by maintaining energy balance and facilitating reproductive processes that require high protein and amino acid availability.

3.4. Antibacterial Screening of *T. gigas* Gut Symbionts

Antibacterial screening was performed on nine symbiotic bacterial isolates obtained from the gut of *T. gigas* using two test bacteria, *E. coli* (Gram-negative) and *S. aureus* (Gram-positive), with incubation periods of 24 and 48hours.

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The screening results showed that the majority of isolates did not exhibit inhibitory activity against the tested bacteria. Clear zones formed by several isolates were limited in size and inconsistent among replicates. Isolates H.A.1.2, H.B.2.3, and H.A.1.5 showed indications of growth inhibition against *E. coli* during the initial screening phase, whereas against *S. aureus*, only isolate H.A.1.2 exhibited a partial positive response. The remaining isolates showed no detectable antibacterial activity.

These findings indicate that the gut symbiont community of *T. gigas* at the study site is not dominated by antibiotic-producing bacteria, but rather plays a greater role in metabolic and enzymatic functions. This condition is commonly observed in gut symbionts adapted to relatively stable environments, where microbial competition is low and selective pressure for antibiotic production is therefore limited (Banerjee & Ray, 2017).

3.5. Antibacterial Activity Test of *T. gigas* Gut Symbionts

Antibacterial activity assays were subsequently conducted on the three isolates that exhibited positive results in the initial screening, namely H.A.1.2, H.B.2.3, and H.A.1.5. Tests were carried out against the same pathogenic bacteria, *E. coli* and *S. aureus*, with observations recorded at 24 and 48 hours (Table 4).

Table 4: Antibacterial Activity Test of Horseshoe Crab Gut Symbionts

Isolate Code	Inhibition Zone (mm)			
	E. coli		*S. aureus*	
	24hours	48hours	24hours	48hours
H.A.1.2	10.22±0.38	9.15 ± 0.91	10.25±1.06	10.10±1.27
H.B.2.3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
H.A.1.5	9.25±0.35	8.10±0.14	8.58±0.91	9.11±1.25
Control (+)	29.27±0.58	29.15±0.49	30.99±0.86	24.61±0.66
Control (-)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

The results of the secondary assays confirmed that isolate H.A.1.2 exhibited the highest antibacterial activity, with inhibition zones against *E. coli* measuring 10.22±0.38mm (24hours) and 9.15±0.91mm (48hours), and against *S. aureus* measuring 10.25±1.06mm (24hours) and 10.10±1.27mm (48hours). Isolate H.A.1.5 also demonstrated relatively consistent activity, with inhibition zones of 9.25±0.35mm (24hours) against *E. coli* and 8.58±0.91mm against *S. aureus*. In contrast, isolate H.B.2.3 failed to maintain antibacterial activity in follow-up assays, which may be attributed to low concentrations of secondary metabolites, degradation of bioactive compounds, or incompatibility between the compound's polarity and the ethyl acetate solvent used (Tumiwa et al., 2019; Jawan et al., 2020). The inhibition zones produced by the symbiont isolates were relatively smaller than those of the positive control (24.61–30.99mm), likely because the tested extracts were still in crude form and may not have been optimally concentrated.

These findings confirm that the gut symbionts of *T. gigas* represent a potential source of secondary metabolites with antibacterial activity that may be utilized in marine biotechnology. Isolates H.A.1.2 and H.A.1.5 are considered primary candidates for further bioactive compound isolation, including chemical characterization and advanced biological activity assays. The results also support the hypothesis that symbiotic bacteria contribute to host biological defense, particularly by reducing the risk of pathogen colonization in the digestive tract. Symbiotic bacteria contribute to host health through the production of antimicrobial compounds that suppress pathogenic microorganisms (Bravo et al., 2022). Nevertheless, these findings indicate that the primary potential of *T. gigas* gut symbionts in this study lies not in serving as sources of strong antibiotics, but rather in contributing to host physiology through enzymatic and metabolic activities.

3.6. Molecular Identification of Bacteria

Molecular identification of two isolates with limited antibacterial activity, namely H.A.1.2 and H.A.1.5, was performed using 16S rRNA gene sequencing. The results showed that isolate H.A.1.2 had a sequence length of 1,385bp (Table 5) and the highest similarity to *Pseudoalteromonas piscicida* (97.48%; query cover 99%; accession number MN096877.1). Meanwhile, isolate H.A.1.5 had a sequence length of 1,490bp (Table 5) and was most closely related to *Vibrio alginolyticus* (94.80%; query cover 100%; accession number MW255191).

Table 5: Blasting Results of Horseshoe Crab Gut Symbionts

Isolate Code	Nucleotide Length	Closest Kinship	Percent Identification	Query Cover (%)	Accesion Number (BLAST NCBI)
H.A.1.2	1385	*Pseudoalteromonas piscicida*	97.48	99	MN096877.1
H.A.1.5	1490	*Vibrio alginolyticus*	94.80	100	MW255191.1

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Both species are marine bacteria commonly found as associated microorganisms in marine invertebrates and are known for their broad metabolic capabilities. *P. piscicida* has been reported to produce various extracellular enzymes and bioactive metabolites, although the expression of antibacterial activity is strongly influenced by environmental conditions and growth phase (Cerezo et al., 2025). In contrast, *V. alginolyticus* is primarily recognized as a heterotrophic bacterium with the ability to degrade complex organic materials, and its antibacterial activity is often strain-dependent and inconsistent (Dhayalan et al., 2025).

The limited antibacterial activity observed in these isolates indicates that the presence of secondary metabolite biosynthetic genes does not necessarily correlate with phenotypic expression under laboratory culture conditions. In marine bacteria, the expression of antibacterial compounds often requires specific induction conditions, such as microbial competition, changes in salinity, or the presence of particular chemical signaling molecules (Svendsen et al., 2024).

3.7. Antibacterial Test of *T. gigas* Body Extract

Antibacterial testing of *T. gigas* body tissue extracts showed that none of the treatments produced inhibition zones against *E. coli* or *S. aureus* after 24 and 48 hours of incubation. These results confirm that the antibacterial activity detected in this study did not originate from host body tissues, but was limited to specific symbiotic microorganisms.

The absence of antibacterial activity in body extracts may be attributed to low concentrations of active compounds, degradation of thermolabile compounds during the extraction process, or the absence of antibacterial metabolites in *T. gigas* tissues outside the hemolymph, which is known to contain immune components such as hemocyanin (Idris & Nadzir, 2021). These findings further emphasize the role of symbiotic microbiota as key functional components in host–microbe interactions.

3.8. Proximate Analysis of *T. gigas* Eggs

Proximate analysis of *T. gigas* eggs revealed that lipids were the dominant component compared to other nutritional fractions. The recorded contents were 7.33±0.47% moisture, 1.04±0.57% ash, 11.57±0.00% lipid, 0.86±0.00% protein, and 1.40±0.00% carbohydrate. This lipid dominance indicates that fats serve as the primary energy source during embryonic development (Table 6).

Table 6: Proximate Analysis

Contents	%
Water	7.33+0.47
Ash	1.04+0.57
Fat	11.57+0.00
Proteins	0.86+0.00
Carbohydrates	1.40+0.00

The high lipid content in *T. gigas* eggs is consistent with the reproductive strategy of benthic marine invertebrates inhabiting fluctuating intertidal environments. Lipids function as a stable and efficient long-term energy reserve to support embryonic development prior to the ability to acquire nutrients from the external environment (Han et al., 2025; Price et al., 2018). The low carbohydrate content suggests that glucose and polysaccharides are not the main energy sources but are mobilized only to a limited extent as components of rapid metabolic processes.

The relatively low protein content in *T. gigas* eggs indicates that structural and enzymatic proteins are likely synthesized gradually during embryogenesis, using free amino acids derived from protein degradation and contributions from maternal reserves. This phenomenon is commonly observed in ancient marine organisms that rely on high metabolic efficiency and energy conservation strategies (Lin et al., 2017; Maldonado-Ruiz et al., 2024).

3.9. Amino Acid Profile Analysis of *T. gigas* Eggs

Analysis of the amino acid profile of *T. gigas* eggs revealed a total amino acid content of 59.32% (w/w), comprising essential and non-essential amino acids in relatively balanced proportions (Table 7).

This composition reflects the eggs' capacity to serve as reservoirs of critical biomolecules that support embryonic growth and early developmental processes. The essential amino acids identified included leucine (5.72%), isoleucine (4.72%), lysine (3.98%), valine (3.97%), phenylalanine (3.48%), threonine (3.39%), histidine (2.30%), arginine (2.92%), and methionine (0.92%). The dominance of branched-chain amino acids (BCAAs), including leucine, isoleucine, and valine, highlights the importance of *T. gigas* eggs in supporting structural protein synthesis and regulating energy metabolism during early embryogenesis. BCAAs are well known as key regulators of the mTOR signaling pathway, which controls cell growth and tissue differentiation (Han et al., 2025).

The non-essential amino acid fraction was dominated by glutamate (7.91%) and aspartate (7.27%), followed by tyrosine (5.29%), serine (3.19%), glycine (1.74%), and alanine (1.71%). Glutamate and aspartate act as major nitrogen donors and precursors for the biosynthesis of other amino acids and play essential roles in energy metabolism and osmoregulation in developing eggs, particularly in coastal environments characterized by high salinity (Lin et al., 2017; Li et al., 2024).

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Table 7: Amino Acid Content

Parameter Amino Acid	Result (% w/w)
Essential	
Leucine	5.72
Isoleucine	4.72
Lysine	3.98
Valine	3.97
Phenylalanine	3.48
Thereonine	3.39
Histidine	2.30
Arginine	2.92
Methionine	0.92
Non-essential	
Glutamate	7.91
Aspartic Acid	7.27
Tyrosine	5.29
Serine	3.19
Glycine	1.74
Alanine	1.71
Amino Acid Total	59.32

The high tyrosine content is particularly noteworthy, as this amino acid serves as an important precursor in the biosynthesis of pigments, hormones, and phenolic bioactive compounds. This finding suggests that *T. gigas* eggs function not only as nutrient reserves but also as active biochemical systems supporting embryonic physiological adaptation to environmental stress.

3.10. Integration of Egg Nutritional Profiles with Gut Symbiont Activity

The high amino acid content observed in *T. gigas* eggs is closely linked to the role of the maternal gut symbiotic microbiota, particularly bacteria with strong enzymatic activities, such as proteases, amylases, and cellulases. Proteolytic activity by these symbionts contributes to the availability of free amino acids through the degradation of dietary proteins and organic tissues, which are subsequently mobilized to reproductive tissues during vitellogenesis.

These findings are consistent with reports by Price et al. (2018) and Han et al. (2025), which indicates that gut microbiota in marine invertebrates actively complement host amino acid biosynthetic pathways and recycle organic nitrogen into functional biomolecules. The presence of symbionts producing hydrolytic enzymes enhances host metabolic efficiency, particularly in ancient organisms such as *T. gigas* that inhabit environments with fluctuating nutrient availability.

Furthermore, the dominance of glutamate and aspartate in the eggs suggests intense metabolic activity, likely influenced by symbiont-mediated nitrogen and carbon cycling. Gut symbionts are known to convert complex organic compounds into simple amino acids and intermediary metabolites that are readily utilized by the host (Li et al., 2024). Thus, the amino acid profile of *T. gigas* eggs can be interpreted as an indirect reflection of the metabolic activity of its symbiotic microbiota.

The integration of symbiont enzymatic data with egg nutritional profiles reinforces the view that the biotechnological potential of *T. gigas* extends beyond the exploration of antibacterial compounds to include highly efficient metabolic mutualism between host and symbionts. This system holds promise for applications in industrial enzyme development, formulation of marine microbial culture media, and conservation strategies informed by the reproductive physiology of ancient marine organisms.

Further studies employing multi-omics approaches, such as metagenomics and metabolomics, are required to identify the genes and metabolic pathways involved in amino acid biosynthesis and the production of hydrolytic enzymes. Such approaches may open new opportunities for the sustainable utilization of *T. gigas* symbionts within the framework of modern blue biotechnology (Maldonado-Ruiz et al., 2024).

4. CONCLUSION

Symbiotic microorganisms associated with the digestive tract of *T. gigas* exhibit diverse enzymatic activities that reflect their important physiological roles in supporting host digestion and metabolism. The presence of amylase, lipase, protease, and cellulase activities in several bacterial isolates indicates that these symbionts contribute to the degradation of organic macromolecules, thereby enhancing nutrient availability within the digestive system. These findings suggest that the primary role of gut symbionts in *T. gigas* is more closely associated with metabolic and nutritional functions than with antibacterial defense mechanisms.

Proximate analysis and amino acid profiling of *T. gigas* eggs revealed a high nutritional value, with a total amino acid content of 59.32%. Essential amino acids such as leucine, isoleucine, lysine, and valine, along with non-essential amino acids including glutamate and aspartate, were present in substantial amounts and play critical roles in embryonic development, tissue formation, and vitellogenesis. The availability of these amino acids is likely linked to efficient nutrient utilization facilitated by symbiont enzymatic activity, which may, in turn, indirectly support reproductive success and early developmental processes in *T. gigas*.

Although antibacterial assays against *E. coli* and *S. aureus* did not demonstrate inhibitory activity, these results provide valuable insight: not all gut symbionts produce detectable antibacterial compounds under *in vitro* conditions. Instead, their functional roles may involve alternative ecological mechanisms, such as nutrient competition, stabilization of the gut microbiota community, and metabolic contributions to the host. Thus, the mutualistic relationship between *T. gigas* and its gut symbionts represents a physiological adaptation to dynamic coastal environments.

Overall, this study provides baseline information on the enzymatic functions of gut symbionts and the

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nutritional characteristics of *T. gigas* eggs, thereby enhancing our understanding of symbiont contributions to host physiology and reproduction. These findings serve as a foundation for future studies employing molecular and omics-based approaches to more comprehensively elucidate symbiont functions, while also highlighting the importance of conserving *T. gigas* as a species with unique biological and ecological significance in coastal ecosystems.

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Data Availability: All data supporting the findings of this study are included within the article.

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