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Detection of non-ribosomal and polyketide biosynthetic genes in bacteria from green mud crab *Scylla serrata* gut microbiome and their antagonistic activities

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ABSTRACT

Multi-modular enzyme complexes known as non-ribosomal peptide synthetases (NRPSs) and polyketide synthetases (PKSs) have been widely reported in bacteria that produce secondary bioactive metabolites such as non-ribosomal peptides (NRPs) and polyketides (PKs), respectively. These NRPS/PKS pathways contribute to synthesizing several antibiotics, such as vancomycin, rifamycin, and bleomycin, which are vital in human medicine. The present study aimed to isolate gut-associated bacteria from mud crab *Scylla serrata*, and detect NRPS and PKS gene clusters associated with it. This study included 36 bacterial isolates from five mud crab gut samples. Biosynthetic gene clusters (NRPS and PKS), were detected by PCR using degenerative primers specific to these genes. Three isolates (FKP2–4, FKP4–1, and FKP2–16) were positive for NRPS and two for PKS (FKP2–4 and FKP4–1) genes. The isolates were subjected to 16S rRNA gene amplification and sequenced. In silico analysis of the sequences using the Basic Local Alignment Search Tool (BLAST) identified the isolates FKP2–4, FKP4–1, and FKP2–16 as *Acinetobacter variabilis, Vagococcus fluvialis,* and *Staphylococcus arlettae*, respectively, after comparing with the existing sequences available in the National Center for Biotechnology Information (NCBI) database. Compared to the control, it was observed that these isolates exhibited intriguing antagonistic activities against *Escherichia coli* and *Staphylococcus aureus*. However, these isolates failed to show significant activity against *Candida albicans*. Exopolysaccharide production by the isolated organisms was tested using Zobell marine agar (ZMA) with 5% sucrose, but none of the colonies were mucoid or slimy.

Introduction

Natural products from microorganisms are structurally diverse and represent a rich source for discovering new drugs to treat various human diseases, including infections and cancer. Bacteria are perhaps the most prolific microbial producers of bioactive natural products, which are represented by their secondary metabolites [1]. Based on their biosynthetic origin, these secondary metabolites can be classified into four groups: polyketides (PKs), non-ribosomal peptides (NRPs), terpenes, and indole alkaloids [2]. NRPs, PKs, and their combinations are the most common amongst them. Their biosynthetic pathways involve enzymes usually encoded by co-regulated genes organized in clusters [3]. Non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) have been reported in bacteria, cyanobacteria, and fungi [4–6]. NRPS/PKS pathways can be used to synthesize several essential human medicines, such as vancomycin [7] and bleomycin [8]. The non-ribosomal and mixed polyketide families account for more than half of all medications used in clinical development [9,10].

Numerous PKs and NRPs were identified in bacteria from terrestrial environments [11]. According to Molinski et al. [12], the oceans, covering more than 70% of the Earth's surface, possess an extensive reserve of valuable natural products. Reddy et al. [13] observed a high diversity of bacterial communities in the marine sediment of the Yellow Sea. They also noted that the biosynthetic genes responsible for natural product production in the ocean differed from those found in soils, indicating the potential of the sea for discovering novel natural

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products. Marine microbes are significant sources of NRPs with various biological activities against bacteria, viruses, and parasites, and they also act as immunosuppressants and animal growth promoters [14]. Specific secondary metabolites in gut bacteria, such as PKs and NRPs, influence the adaptation and survival of these microorganisms under adverse environmental conditions. Antibiotics, anticancer agents, immunosuppressants, toxins, and siderophores are multi-domain mega--enzymes known as PKS and NRPS [15].

The mud crab *Scylla serrata* is the most extensively distributed species in estuaries, coastal areas, habitats, and soft muddy bottoms. The microbiota of the digestive system is controlled by genetics, environment, nutrition, and other factors, and the intestinal flora is vital for maintaining host health [16]. Crabs obtain bacteria from the gut through water and food. The gut environment provides favorable conditions for the growth of microorganisms [17], which play a significant role in immune responses and disease resistance because the bacteria synthesize highly resistant peptides against infectious microbes [6]. Gut microbiota is vital in host gastrointestinal tract development, nutritional status, immune responses, and disease resistance [18,19]. Since PKs and NRPs derived from microorganisms exhibited similarities to those documented before, it would be an excellent decision to investigate animals, like crabs, and their gut microbiome for new bioactive substances.

Detecting NRPS/PKS-positive microorganisms in the gut microbiome of invertebrates, especially crabs, is highly appreciated because crabs adapt to living in both land and aquatic habitats. As they are exposed to extreme environments, it is possible to explore novel bioactive compounds that may exhibit a natural resistance to most pathogens. It has been reported that the actual producers of many drug candidates isolated from invertebrates are symbiotic bacteria [20–22]. Since the emergence of antibiotic-resistant bacteria has become a significant concern in the medical/healthcare sector, mining novel bioactive compounds from natural sources is in great demand. Hence, this study focused on detecting natural antimicrobial biosynthetic peptides, such as non-ribosomal and polyketide peptides, from the gut-associated bacterial community of mud crabs, followed by their antimicrobial activities.

Materials and methods

Sample collection and isolation of gut-associated bacteria

Live green mud crabs ($n = 3$) were purchased from the Kundapura fish market in Mangalore, where the wild-caught crabs were locally available. Crabs were placed in a perforated plastic basket, sprinkled with sterile water, and transported to the institute. Before dissecting the crabs in the lab, they were subjected to cryoanesthesia, followed by cleaning with sterile water and disinfection with 70% ethanol. The gut was aseptically removed using a sterile blade, and portions of the intestine were minced into small pieces and transferred into sterile 15 ml Falcon tubes. The samples were serially diluted with sterile physiological saline after homogenizing the tissues using a mortar and pestle. 10^{-5} and 10^{-6} Dilutions were poured into Petri dishes, adding approximately 20 ml of molten Zobell marine agar (ZMA; HiMedia, India). The plates were incubated at 30°C for 24–48 h. Morphologically distinct bacterial colonies were isolated, purified on ZMA plates, and stored in a refrigerator for further use.

Bacterial crude DNA preparation

Crude genomic DNA was prepared as described by Divyashree et al. [23]. Briefly, single colonies of the respective bacterial cultures were inoculated in 5 ml LB broth and incubated for 18 h in a rotary shaker. Then, 1 ml of the culture was taken in a 1.5 ml microcentrifuge tube and centrifuged at 5000 rpm for 10 min. The supernatant was discarded, and the pellet was dissolved in 300μl 1X TE buffer. The tubes were placed in a dry bath at 95 °C for 10 min and immediately cooled on ice. Next, the tubes were spun at 5000 rpm for 5 min, and the freshly collected DNA was kept at −20°C for PCR.

Screening of NRPS and PKS genes by PCR

A thermal cycler (Eppendorf Nexus GX2) was used for PCR with gene-specific primers (Table 1) for the NRPS and PKS genes in the gut-associated bacterial strains. After PCR, the products were run on a 1.5% agarose gel with a 100 bp DNA marker, stained with ethidium bromide (0.5 mg/ml), and viewed under a gel documentation system (Bio-Rad, USA).

Molecular identification of NRPS/ PKS positive bacterial isolates by 16S rRNA gene sequencing

NRPS/PKS-positive bacterial isolates were subjected to PCR, using the 16S rRNA gene primer pairs listed in Table 1. The PCR products were purified using a QIAqick PCR Purification Kit (Qiagen, Germany). Samples were outsourced for sequencing to Eurofins Genomics India Pvt. Ltd., Bangalore, India.

Phylogenetic tree construction and analysis

The obtained sequences were aligned and edited using the MultAlin online program (http://multalin.toulouse.inra.fr/multalin/). Next, sequence similarity and identification were performed using NCBI-BLAST to identify the closest GenBank relatives. The evolutionary history of the identified strains was reconstructed using the neighbor-joining method, which is based on the Maximum Composite Likelihood substitution model [28]. Molecular Evolutionary Genetics Analysis version 10 (MEGA X) bioinformatics tool was used [29] to generate a phylogenetic tree to determine similarities between the query sequence and NCBI sequences.

Antimicrobial assay

Antimicrobial bioassays were performed using the paper-disk plate method with *E. coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), and *Candida albicans* (MTCC 227) as reference organisms. Sterile filter paper disks (6 mm in diameter; HiMedia, India) were wetted with 50 μl of isolate culturesand placed on top of the plate lawned with indicator organisms. Plates were observed for 24–72 h for the bacterial indicators and *C. albicans*. The diameter (mm) of the clear inhibition zone around each paper disk was measured.

Exopolysaccharide production

The bacterial isolates were streaked onto ZMA supplemented with 5% sucrose as an additional carbon source and incubated at 30°C for 48 h. Cultures producing thick mucoid, ropy-like colonies were considered exopolysaccharide producers [30].

Results and discussion

Antimicrobial resistance (AMR) is a severe global threat to humans, animals, and the environment, mainly because of the emergence, spread, and persistence of multidrug-resistant bacteria [31]. The primary reasons for this resistance are the excessive use of antibiotics in food, pets, aquatic animals, and humans; over-the-counter distribution of antibiotics; poor sanitation; and the release of non-metabolized antibiotics or their residues into the environment through manure/feces [32]. The novel bioactive compounds extracted from the natural sources have high antimicrobial activity against multidrug- resistant bacteria [33]. Cragg et al. [34] reported that microbial origin accounts for 80% of antimicrobial drugs used in the pharmaceutical industry. However, the

Table 1
Primers used for this study.

Target gene	Primer ID	Sequence (5′ - 3′)	References
NRPS adenylation domain fragments	AD_F/R	F-CGCGCGCATGTACTGGACNGGNGAYYT R- GGAGTGGCCGCCARNYBRAARAA	[24]
NRPS adenylation domain fragments	A3F/ A7R	F-GCSTACSSYSATSTACACSTCSGG R- SASGTCVCCSGTSCGGTAS	[25]
Type I polyketide synthase KS domain fragments	PK_1F/1R	F-GGCAACGCCTACCACATGCANGGNYT R- GGTCCGCGGGACGTARTCNARRTC	[24]
Type I polyketide synthase KS domain fragments	KSDPQQ_F/ KSHGTG_R	F-MGNGARGCNNWNSMNATGGAYCCNCARCANMG R- GGRTCNCCNARNSWNGTNCCNGTNCCRTG	[26]
16S rRNA	27 F/ 1492 R	F- AGAGTTTGATCCTGGCTCAG R- GGTTACCTTGTTACGACTT	[27]

extracted antimicrobial biosynthetic genes (PKs and NRPs) from cultured microbes appear identical. Therefore, it seems significant to investigate diverse sources to discover novel natural antimicrobial biosynthetic peptides, such as non-ribosomal and polyketide peptides.

Isolation of gut-associated bacteria and screening of NRPS/ PKS gene clusters by PCR

The gut portion of the crabs was dissected, homogenized, serially diluted, and plated onto ZMA plates. After 24 h of incubation, 50 morphologically distinct colonies were isolated. After purification, only 36 strains (contaminated ones were discarded) were subjected for PCR screening of NRPS/PKS genes, and 12 showed as positive for NRPS and PKS genes. Finally, six were selected for sequencing because they consistently yielded single bands in the respective gene amplifications (Table 2).

Xie et al. [35] reported that the wild *S. paramamosain* were found to have a greater diversity and bacterial load, revealing the role of environmental factors in forming the gut microbiome. Their study identified Bacteroidetes, Actinobacteria, Firmicutes, Proteobacteria, and Cyanobacteria as the most significant phyla in the *S. serrata* gut microbiome. *Fusobacteria* and *Tenericutes* were among the core gut microbiome phyla of *S. paramamosain*. Benny et al. [36] found that because of its sterility, the hemolymph of marine crabs is the most promising resource for combating pathogens rich in NRPS gene clusters. The study discovered that the hemolymph of marine crabs comprises several species, including *Bacillus albus, Bacillus megaterium, Staphylococcus saprophyticus, S. sciuri, S. haemoliticus and S. arlettae*.

Dong et al. [16] studied the microbial population and expression of immune-related genes in the gut of Chinese mitten crabs. They discovered that bacterial diversity is higher in the hindgut, similar to the expression of antimicrobial peptides and immune genes, such as EsRelish (IMD pathway). They discovered that microbiota and bacterial communities in crab digestive tracts are site-specific, and that intestinal immunity and microbiota are closely associated.

Li et al. [37] analyzed the bacterial communities in the intestines of wild crabs, pond-raised healthy crabs, and diseased crabs from the bacterial populations in the intestines of the mud crab *Scylla paramamosain* and found that those populations were distinguishable.

Molecular identification of the strains

The 16S rRNA gene of the six bacterial isolates that showed positive for NRPS/ PKS was PCR amplified, and the products (~1500 bp) were sequenced. The sequences were edited using the MultAlin interface page bioinformatics approach, and the final sequences were analyzed. Accession number, query cover, and percentage of similarities with the organisms deposited in NCBI were recorded using BLAST. After the sequence analysis, three strains, FKP2-4, FKP2-16 and FKP4-1 showed maximum coverage with the reference strains in NCBI were chosen for further studies and identified as *Acinetobacter variabilis, Staphylococcus arlettae*, and *Vagococcus fluvialis,* respectively. The evolutionary tree exhibited 98–100% similarity to these strain types (Table 3). The NCBI accession numbers of the sequences obtained for these organisms were OP090353, OP090355, and OP090357, respectively (Supplementary files: S3-S8).

Acinetobacter variabilis has been majorly reported to recover from human clinical specimens as well as feces of cattle [38]. There were no reports of its presence in the marine environment. *Staphylococcus arlettae* has been isolated from different animals and environments, including salt mines, estuaries, fermented foods, and biological safety cabinets. Highly salt-tolerant strains of *S. arlettae* are also reported and are commonly found in the marine environment [39,40]. *Vagococcus fluvialis* is found in various environments including mammals, fish, birds, rivers, seawater, and sponges [41].

A phylogenetic tree (Fig. 1) was constructed using the 16S rRNA gene sequences of the three isolates (FKP2–4, FKP2–16, and FKP4–1) and other reference bacterial strains available at NCBI. MEGA X was used to estimate the evolutionary origin using the Maximum Composite Likelihood method based on the neighbor-joining statistical model. The FKP2–4 sequence displayed 98.27% similarity to the query sequences of *Acinetobacter* species, including *A. variabilis* and *A. calcoaceticus*. However, it predominantly resembled *A. variabilis*, identifying the FKP2–4 isolate as *A. variabilis* (Supplementary file S6). Regarding the FKP2–16 sequence, the first 25 BLAST hits exhibited similarity ranging from

Table 3
NCBI-BLAST analysis of 16S rRNA gene sequences of the isolates.

Isolate ID	Closest relative sequences of the amplicon in NCBI	Accession number	Identity (%)	GenBank number of the isolate
FKP2–4	*Acinetobacter variabilis*	MN932362	98.27	OP090353
FKP2–16	*Staphylococcus arlettae*	JX188024	98.65	OP090355
FKP4–1	*Vagococcus fluvialis*	MT103047	100	OP090357

Table 2
Overall result (PCR, antimicrobial and exopolysaccharide activities).

Bacterial strains	NRPS	PKS	Resistance to *E. coli*	Resistance to *S. aureus*	Resistance to *C. albicans*	Exopolysaccharide production
Acinetobacter variabilis	+	+	+	+	–	–
Vagococcus fluvialis	+	+	+	+	–	–
Staphylococcus arlettae	+	–	+	+	–	–

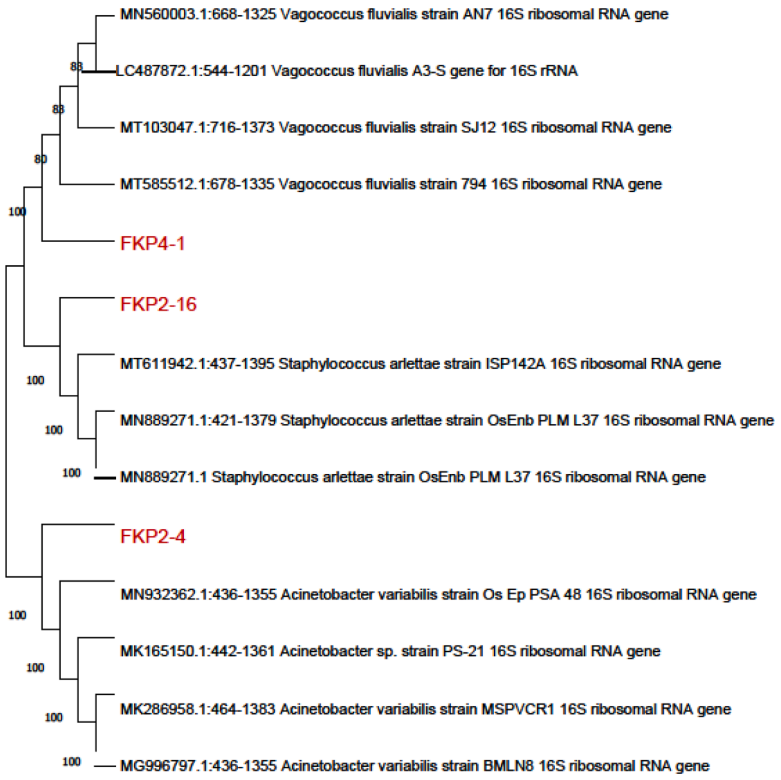

Fig. 1. Neighbor-joining phylogenetic tree of 16S rRNA genes of NRPS- and PKS-positive strains from mud crabs. The sequences used in the present study are boxed [FKP2–4_ *Acinetobacter variabilis*, FKP2–16_ *Staphylococcus arlettae*, FKP4–1_ *Vagococcus fluvialis*]. Numbers adjacent to the names of the organisms represent their accession numbers, and the numbers near the nodes in the tree represent bootstrap values from 1000 replicates.

98.54% to 98.65% to the query sequences of *Staphylococcus arlettae* (Supplementary file S7). Likewise, for the FKP4–1 sequence, the BLAST hits demonstrated a 100% similarity to *Vagococcus fluvialis* (Supplementary file S8). The tree indicates the division of these sequences into two groups. *Acinetobacter* species were present in one group, and *Staphylococcus* and *Vagococcus* species belonged to another group. The tree visually represents the evolutionary relatedness of these bacterial species and provides insights into their genetic connections.

Apine et al. [42] conducted a study comparing the gut microbiome composition, including species richness and abundance, of *S. serrata* from both wild and farmed locations on India's east and west coasts. They found that the water temperature significantly impacted the gut microbiome composition, leading to a reduction in microbial diversity as the water temperature increased. Additionally, their study suggested that farming practices did not significantly impact the gut microbiome composition of the crabs compared to wild-caught crabs.

For the first time, Zote et al. [43] reported the presence of the antimicrobial biosynthetic genes PKS type II, NRPS, and the CYP pathway in crab-associated bacteria. The bacterial population associated with mud crabs shows significant antimicrobial activity and the presence of various volatile agents, which are potential sources of antimicrobial agents.

In a study conducted by Soundarapandian and Sowmiya [44], the gut microbiome of two economically important crabs, *Portunus sanguinolentus* and *P. pelagicus*, was examined. They discovered the presence of both gram-positive and gram-negative bacteria in the gut. It was observed that gram-negative bacteria tended to be the predominant forms in the gut samples overall. Specifically, *Vibrio parahaemolyticus, Pseudomonas fluorescens, Staphylococcus aureus,* and *S. saprophyticus* were identified as the dominant forms. This study identified different strains, specifically, *Acinetobacter variabilis, Staphylococcus arlettae,* and *Vagococcus fluvialis* in the mud crab gut samples.

In the present study, the NRPS and PKS- positive bacterial strains in the gut of mud crabs consisted of gram-positive and gram-negative bacterial species such as *Staphylococcus, Vagococcus,* and *Acinetobacter*, respectively. In this study, both *A. variabilis* (gram-negative) and *V. fluvialis* (gram- positive) were positive for both NRPS and PKS. Sivasubramanian et al. [17] also reported gram-positive and gram-negative bacteria, such as *Photobacterium, Vibrio, Flavobacterium, Bacillus, Pseudomonas, Aeromonas, Alcaligenes,* and *Staphylococcus, Enterobacter, Micrococcus, Corynebacterium, Flavobacterium,* etc. in the crab gut.

Antimicrobial assay

The antagonistic activities of the test organisms against *E. coli* and *S. aureus* were observed for 24–48 h, and all test cultures showed zones of inhibition around the disks. The clearing zones ranged from 10 to 14 mm in all tested organisms, compared to the control disks (Table 2; Supplementary files: S1 & S2). The bacterial strains tested against *C. albicans* showed no significant activity after 72 h of incubation.

Antagonistic activity is one microorganism's ability to inhibit another organism's growth or activity. Marine bacteria can exhibit antagonistic activity towards other microorganisms, including bacteria, fungi, and viruses. This activity can be attributed to producing various secondary metabolites, such as antibiotics, enzymes, and bioactive compounds, with potential applications in developing new drugs and therapies for multiple diseases. The antimicrobial substances produced by microorganisms include organic acids, diacetyl, hydrogen peroxide alone or in combination, biocides, probiotics, and sterilants [45,46]. In this study, *A. variabilis, S. arlettae,* and *V. fluvialis* exhibited antagonistic activities against standard *E. coli* and *S. aureus* but no activity against the fungal pathogen *C. albicans*. Shtenikov et al. [47]. reported isolating three aerobic, spore-forming bacilli of genera *Bacillus, Priestia,* and *Paenibacillus* from deep-sea bottom sediments of the Black Sea that exhibited antagonistic activities against standard strains of *E. coli* and

S. aureus. Patel et al. [48] isolated nine pigment-producing bacterial strains from Arabian Sea water samples, and four isolates (NP5, NP6, NP8 & NP9) among them showed good antibacterial activity against the different bacterial cultures (*Bacillus cereus, E. coli, Vibrio cholera, Bacillus subtilis, Staphylococcus aureus,* and *Bacillus megaterium*). Among these four isolates, NP9 showed the highest antibacterial activity against all test cultures and was later identified as *Candidatus chryseobacterium massiliae*.

Exopolysaccharide production

The marine organisms surviving in extreme ecological conditions develop new adaptive strategies, including synthesizing secondary metabolites, to continue to exist in the surrounding microenvironment [31, 33,49]. Among these metabolites, exopolysaccharides play essential physiological roles in protecting cells from desiccation, predation, osmotic stress, and antimicrobial effects [50]. As a result, these polysaccharides have various industrial applications, including biosorbents, binders, coagulants, emulsifiers, stabilizers, gelling agents, thickeners, and viscosifiers [51].

Microbial exopolysaccharides are biopolymers that are secreted by microbial cells as loosely bound slimes associated with the cell surface [52]. The quantity and composition of microbial EPS produced depend on the species and culture conditions of the organism, as shown by several studies [53]. The composition of EPS has a variety of organic and inorganic substances with structural variables like either homopolysaccharides such as dextran, mutan, and levan or heteropolysaccharides [54].

Poli et al. [50]., in a review, enlisted exopolysaccharide-producing various bacterial species isolated from different marine environments, such as microorganisms isolated from marine hot springs and hydrothermal vents (species of *Pseudoalteromona, Alteromonas, Thermococcus, etc.*), cold marine environments (species of *Pseudoalteromonas, Colwellia psychrerythraea* etc.), and hypersaline marine environments (species of *Haloferax, Hahella, Halomonas, etc.*). However, in our study, none of the cultures showed signs of mucus or slime production on the ZMA plates after overnight incubation. In our study, the test strains were streaked onto ZMA supplemented with 5% sucrose as an additional carbon source and incubated at 30°C for 24 h. Colonies were well isolated, but none exhibited any signs of mucous or slimy nature after overnight incubation.

Conclusion

The bacterial strains isolated from the gut of mud crab *Scylla serrata* have the potential to produce bioactive secondary metabolites with antagonistic, antimicrobial, or biological properties because of the presence of antimicrobial biosynthetic gene clusters such as NRPS and PKS. To analyze the antagonistic activity against pathogenic microbes, the antimicrobial properties of these bacterial strains should be investigated as they were positive for biosynthetic gene clusters. Hence, the NRPS/PKS genes will be cloned for sequencing and characterized those genes for their antimicrobial properties using in-silico approaches such as the discovery of the domain 'A,' consisting of conserved motifs. Our future goals will be the expression and characterization of these, as there has been no definitive research on NRPS/PKS diversity in the gut microbiota of crabs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Data availability

Data will be made available on request.

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Ethics Statement

The mud crab *Scylla serrata* is not considered an endangered or protected species, and it is widely cultivated in aqua farms and available in the market from wild catch and crab farms. Therefore, no specific authorization was required to work on mud crab used in this study.

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Author contributions

Madhu Mani: Conceptualization, Methodology, Supervision, Reviewing and Editing. Shabreen Banu: Experiments, Analysis, Result Interpretation, Writing- Original draft preparation. Shivakiran Alva: Antimicrobial Studies. Prathiksha Prabhu: Antimicrobial Studies. Sreedharan Krishnan: Final Reviewing and Editing.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fsirep.2023.100104.

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