

Full Length Research Paper

Isolation of gut associated bacteria from mangrove crabs collected from different mangrove regions of Tamil Nadu, South east coast of India

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Mangrove crabs are mostly herbivorous which consume more amount of leaf litter of various mangrove species and also plays most important role in leaf degradation. Several studies reported that crab harbor bacteria from the environment through water and food. Bacterial species of the gut can influence the health and robustness of the host. The present study aims to isolate and enumerate the bacterial count from the gut of crabs collected from different mangrove environments. The results shows that maximum bacterial load was recorded in *Sesarma Brockii* crabs gut collected from Pichavaram mangroves and minimum was observed in *Metopograpsus maculatus* crabs collected from Uppanar estuarine mangrove. In the same way, maximum bacterial load was observed in both water and sediment samples of Pichavaram. Bacteria belonging to the genera *Bacillus*, *Pseudomonas* and *Aeromonas* were found at higher levels in all the different mangrove regions. In conclusion, crabs in the various mangrove environments carry a particular bacterial flora, which reflects their environment. The Pichavaram mangrove ecosystem is endowed with a high bacterial load due to the continuous shedding of foliage into the water and subsequent decomposition than other mangrove environments. SEM results confirm that crowded populations of bacteria were attached to the gut region of the mangrove crabs.

Key words: Mangrove crabs, gut microflora, isolation, characterization, sediments.

INTRODUCTION

Several species of mangrove crabs are recognized as herbivorous and can consume a good quantity of leaf litter of different mangrove species. These crabs play a key role in the process of leaf degradation and making mangrove leaves more rapidly available to meiofauna

(Ravichandran et al., 2007b). Crab receives bacteria in the gut from their aquatic environment through water and food that are populated with bacteria. Being rich in nutrient, the environment of the crab gut confers favourable conditions for the microorganisms.

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The intestine microbial diversity has been studied in a wide range of aquatic animals (Liu et al., 2011).

The importance of gut bacteria in the nutrition and well being of their hosts has been established for homeothermic species, such as birds and mammals. It is clear that bacterial species of the gut can influence the health and development of the host. Extreme examples of the influence of the gut flora embrace the negative effects of pathogenic organisms and in contrast, the total reliance that ruminants have on their gut flora for the assimilation of organic carbon from the environment (Kennedy et al., 1991). An understanding of the host intestinal bacterial floral interactions is of much significance for the development of a healthy cultivation environment and also to optimize the possible species growth in aqua culture (Oxley et al., 2002). Recent studies suggest that the predominant rod-shaped bacteria in the hindgut are tightly attached to the epithelium surface by an unusual pili-like structure (Chen et al., 2015). Several studies reported on gut flora of marine crabs which includes *Callinectes sapidus* (Huq et al., 1986), *Macrocheira kaempferi* (Ueda et al., 1989), *Eriocheir sinensis* (Li et al., 2007), *Scylla serrata*, *Scylla tranquebarica*, *Portunus pelagicus*, *Portunus sanguinolentus*, *Charybdis helleri* (Ravichandran and Kannupandi, 2005; Rameshkumar et al., 2009) and reports also includes on pathogenic bacteria isolated from gut of *Portunus pelagicus* (Talpur et al., 2011). Despite these reports, there is still lack of similar information on crab species which inhabit mangrove environment. Hence, the present study was designed to isolate and enumerate the total bacterial population from fifteen different mangrove crabs species which were collected from various stations mangrove environments and also examined the gut associated bacteria using scanning electron microscope (SEM).

MATERIALS AND METHODS

Sample collection

From five different mangrove regions including Muthupet, Pazhayar, Pichavaram, Vellar and Uppanar, crabs were collected during the period of November 2013 to October 2014. Fifteen crab species including *Heteropanope indica*, *Macrophthalmus depressus*, *Metapograpsus maculatus*, *Metapograpsus messor*, *Nanosesarma batavicum*, *Nanosesarma minutum*, *Neoepisesarma mederi*, *Neoepisesarma tetragonum*, *Pseudograpsus intermedius*, *Sesarma andersoni*, *Sesarma bidens*, *Sesarma brockii*, *Sesarma plicatum*, *Uca annulipes* and *Uca triangularis* were collected and each species contain 25 individuals taken for the present study. Sterile forceps were used to pick the crabs and crab species were collected immediately and transferred to sterile polythene bags. All the samples were transported immediately to the laboratory and subjected to several analysis. Water and sediment sample were collected from all the five stations using sterile bottles and sterile polythene bags (approximately, 100 ml of water and 100 g of sediment from each site) using sterile spatula.

Segregation of the gut

Prior to segregation of the gut of each crab, species were bathed in 10% formalin for 30 s. Again, the crabs were washed with tap water for 5 min and finally with sterile de-mineralized water in order to remove surface micro flora. Sterilized dissecting materials were used for this study. Crab specimens were dissected and the whole gut was removed (Talpur et al., 2011).

Enumeration of total bacterial load

Gut sample

To avoid individual variations of the gut microflora (Spanggaard et al., 2000), the gut of 15 species of crabs were pooled by species and homogenized in 10 ml of a sterilized nine-salt solution (NSS) (Olsson et al., 1992). Gut homogenates and water sample were diluted in NSS up to ten fold times and appropriate dilutions were spread on the surface of Zobell marine agar (ZMA) plates (Hi-media, Mumbai) in triplicates. The plates were incubated at 28°C for 24 – 48 h. The microbial load was counted and mentioned as the number of colony forming units (CFU).

Water and sediment sample

From the collected water sample, 1 ml of water sample was pipetted out using a sterile pipette into a 9 ml blank and shaken well. From this, 1 ml was pipetted out and added to the 9 ml blank, likewise the serial dilutions were made upto sixth dilutions and used as inoculum. From the sediment sample, 1 g of sediment from each station was transferred aseptically to a 99 ml blank. The contents were homogenized for 10 min. From this, 1 ml was transferred aseptically to a 9 ml blank and mixed thoroughly. Likewise, serial dilutions were made and used as inoculum. Appropriate dilutions were spread on the surface of ZMA plates (Hi-media, Mumbai) in triplicate. The plates were incubated at 28°C for 24 - 48 h. The microbial load was counted and it was recorded as the number of CFU.

Characterisation of isolated bacteria

The isolates were characterised by Gram staining and biochemical test includes motility, oxidase activity, catalase activity, oxidation/fermentation, glucose acid, glucose gas, pigment production and citrate utilization. A series of secondary tests were used (indole, methyl red, Voges-Proskauer, citrate utilization, triple sugar iron, urease, lactose fermentation nitrate reduction, catalase, oxidase and starch hydrolysis tests), when required, to complete the genus level confirmation of isolates. 0% sodium chloride media tests were also included.

Scanning electron microscopic studies

Immediately after dissection, gut of the crabs were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, for 3 h. Samples were washed three times in sodium cacodylate buffer post fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate for 1 h, and washed another three times before dehydrating in a series of ethanol dilutions. Before examining, samples were mounted on aluminium stubs, coated with gold palladium by critical point and dried using carbon dioxide as the transitional fluid, and examined in a JEOL JSM-840 scanning electron microscope (SEM).

Table 1. Number of isolated bacterial genus from the mangrove crabs.

Mangrove crab	<i>Acinetobacter</i>	<i>Bacillus</i>	<i>Enterobacter</i>	<i>Vibrio</i>	<i>Alcaligenes</i>	<i>Photobacterium</i>	<i>Pseudomonas</i>	<i>Aeromonas</i>	<i>Flavobacterium</i>	<i>Staphylo coccus</i>	Total
<i>Heteropanope indica</i>	3	6	2	2	5	2	2	2	0	3	27
<i>M. messor</i>	2	5	2	4	2	2	4	5	1	3	30
<i>Macrophthalmus depressus</i>	4	5	3	3	4	2	1	1	0	2	25
<i>Metapograpsus maculatus</i>	1	7	5	3	5	6	2	2	2	1	34
<i>N. batavicum</i>	2	6	2	3	2	1	4	3	1	3	27
<i>N. mederi</i>	4	9	1	5	2	4	6	7	0	1	39
<i>Nanosesarma minutum</i>	2	5	2	2	2	5	4	1	2	0	25
<i>Neoepisesarma tetragonum</i>	2	6	1	2	3	2	4	5	0	0	25
<i>Pseudograpsus intermedius</i>	4	8	3	3	5	2	4	6	4	2	41
<i>S. bidens</i>	1	9	2	5	1	3	4	4	1	0	30
<i>S. brockii</i>	6	14	5	2	5	5	9	8	4	0	58
<i>S. plicatum</i>	3	9	3	4	4	5	8	8	2	0	46
<i>Sesarma andersoni</i>	2	10	3	2	3	4	5	1	0	2	32
<i>U. triangularis</i>	2	6	5	5	3	2	4	3	2	7	39
<i>Uca annulipes</i>	4	8	3	3	6	4	4	6	1	6	45
Total	42	113	42	48	52	49	65	62	20	30	523

RESULTS

Total bacterial population

Gut sample

The numbers of cultivable bacterial cells present in crab gut were estimated after isolation and growth on ZMA plates incubated at room temperature at 28°C. The total heterotrophic bacterial load ranged between $0.7 \pm 0.49 \times 10^6$ and $8.9 \pm 0.13 \times 10^6$ CFU/g of crabs gut sample and it was found to be the maximum ($8.9 \pm 0.13 \times 10^6$ CFU/g) in *S. brockii* gut collected from Pichavaram mangrove region and minimum ($0.7 \pm 0.49 \times 10^6$) in *M. maculatus* collected in the mangrove region of Uppanar. Between the crab species, the highest bacterial load was noticed in *S. brockii* in all the five stations of Tamil Nadu

(Table 1) and between the stations crabs collected from Pichavaram showing the maximum bacterial populations followed by Vellar, Pazhiyar, Muthupet and Uppanar (Figure 1).

Water and sediment sample

Total bacterial load of water from five mangrove regions were enumerated. Among the five stations, a high bacterial load was observed in the sample collected at Pichavaram mangrove region ($7.2 \pm 0.37 \times 10^6$) and minimum of $4.2 \pm 0.51 \times 10^6$ was observed in Uppanar estuarine mangrove (Figure 2). Similarly, in sediment samples, maximum bacterial load was observed in Pichavaram mangrove region ($9.4 \pm 0.51 \times 10^6$) followed by Vellar ($6.5 \pm 0.29 \times 10^6$), Pazhiyar ($6.3 \pm 0.49 \times 10^6$), Muthupet ($6.0 \pm 0.51 \times 10^6$) and

Uppanar estuary ($5.3 \pm 0.81 \times 10^6$), respectively (Figure 1). Two-way ANOVA showed significance at 0.05% level between all the stations (Table 1).

Characterisation of isolated bacteria

A total of 523 bacterial strains were isolated from gut of fifteen mangrove crab species collected from five different stations and classified into ten taxonomic groups including highest number of followed by *Acinetobacter* (42), *Aeromonas* (62), *Alcaligenes* (52), *Bacillus* (113) *Enterobacter* (42), *Flavobacterium* (20) *Photobacterium* (49), *Pseudomonas* (65), *Staphylococcus* (30) and *Vibrio* (48), (Table 1). All the isolated bacteria were tested for their biochemical characters which showed 89% of the isolates were Gram negative bacilli and 6% were Gram negative cocci, 4%

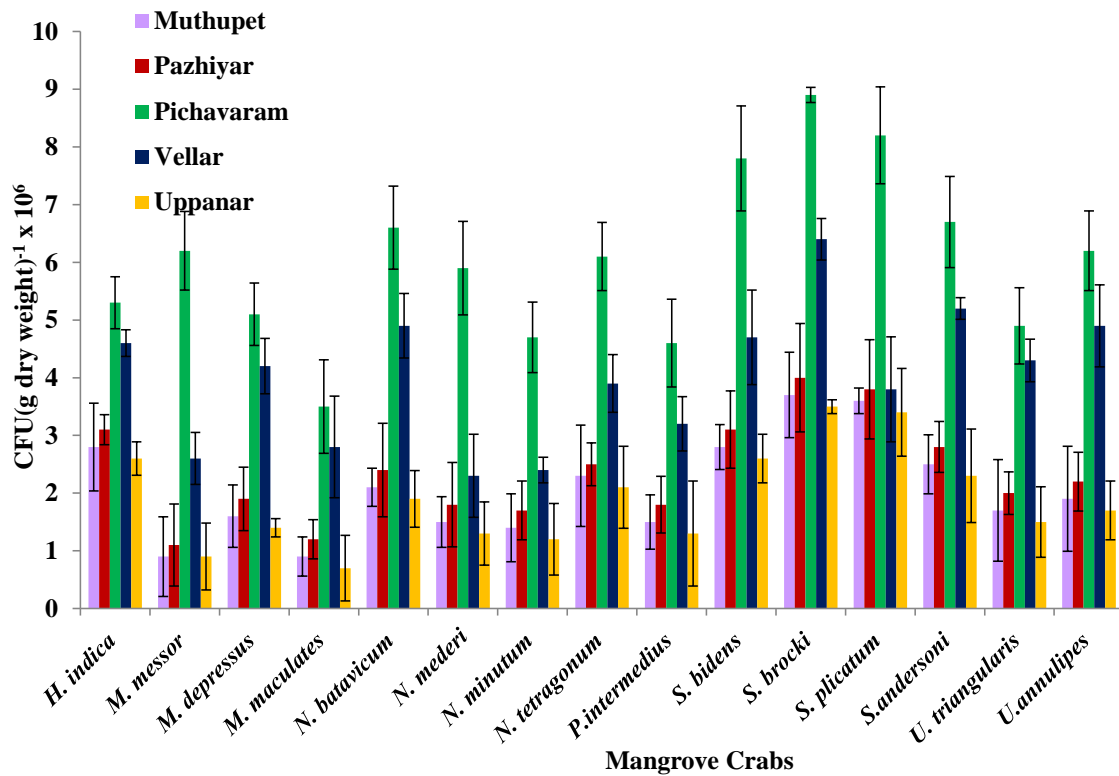


Figure 1. Total bacterial load in the gut of various mangrove crabs from five various stations.

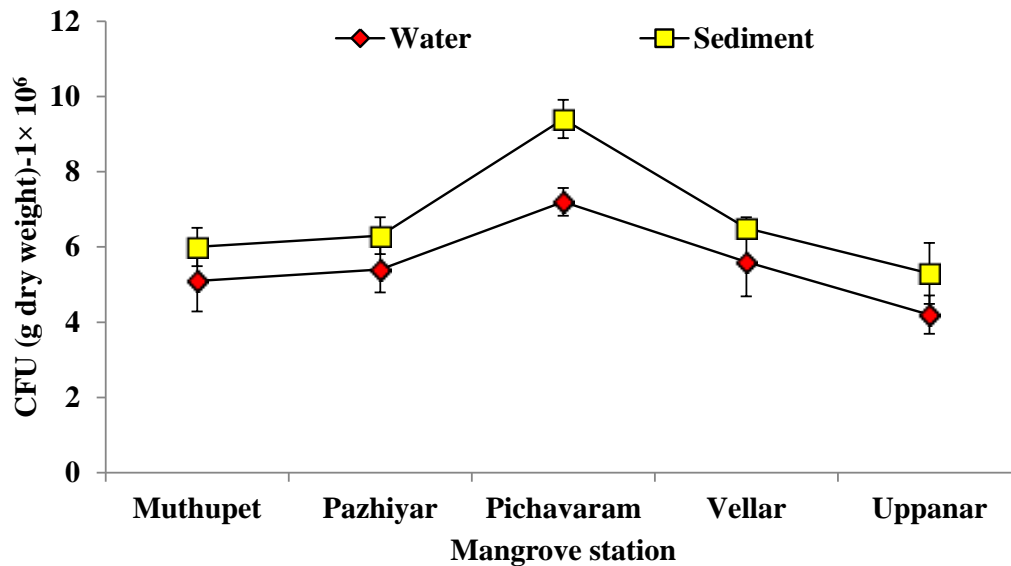


Figure 2. Total bacterial load from five mangrove environments.

were Gram positive bacilli and the remaining 1% was Gram positive cocci. The bacterial isolate showed 40% utilized the citrate and 20% strains were positive in indole

and also in H₂S production test. Methyl red test and lactose fermentation showed positive on 50% of the isolates. 30% of the isolates showed positive results on

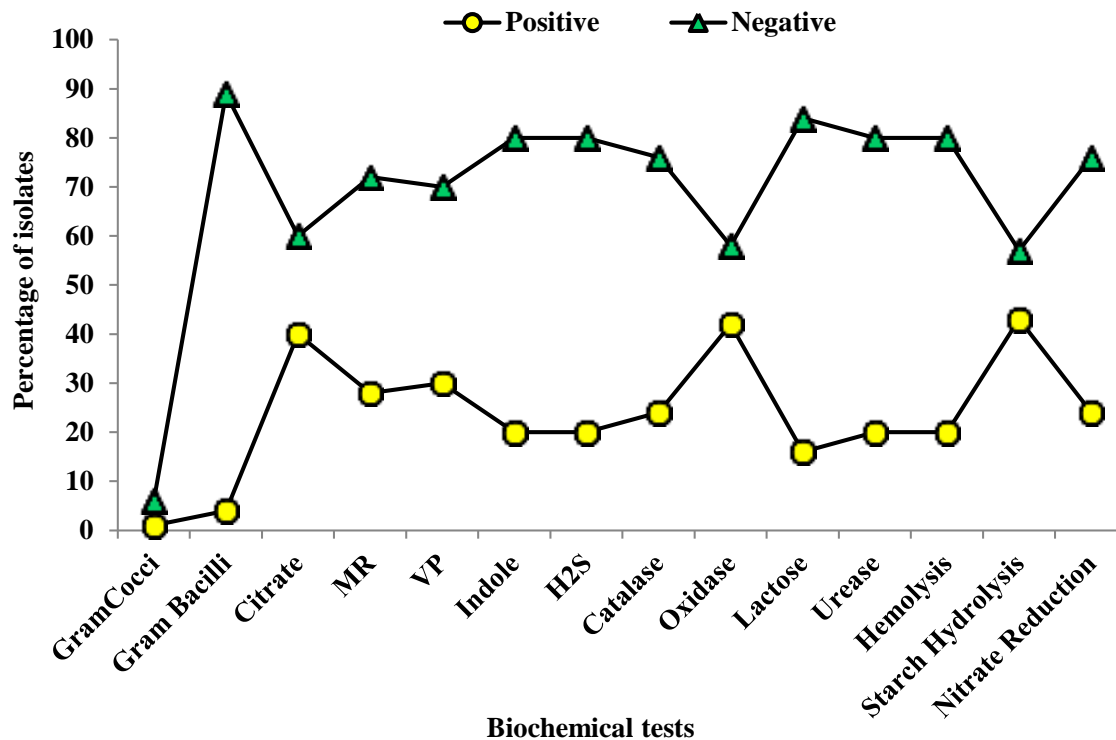


Figure 3. Biochemical characteristic of bacteria isolated from various mangrove crabs.

VP test and 20% of the isolated strains showed positive results on urease test. With regards to catalase character, about 24% of strains showed the positive results and 42, 43 and 24% of the strains showed positive activity in oxidase, starch hydrolysis and nitrate reduction analysis, respectively (Figure 3).

Scanning electron microscopic studies

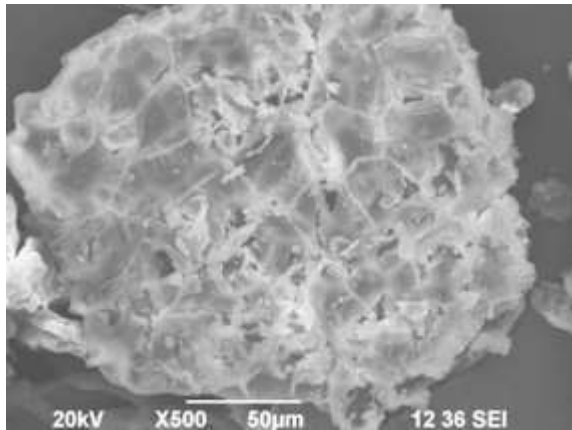
Dense populations of apparent bacteria attached to gut regions of all the fifteen crabs were observed by SEM (Figure 4A to O). SEM study showed that common bacterial morphologies are rods and cocci form. Bacteria neither colonize all surfaces nor all projections in the pyloric stomach, but appeared restricted to chitinous pyloric finger like projections, where they colonized from base to tip with approximately uniform density. Rod shaped bacterial cells were attached to the fingerlets, images of semi-thin sections revealed coccoid bacteria in the gut of inspected crabs. At least two distinct bacterial morphologies colonized the hindgut lining, each consistently observed within a specific region. Curved, rod-shaped bacteria were associated with the anterior hindgut region. Dense aggregations of rod-shaped bacteria colonized the posterior region. hindgut region. Dense aggregations of rod-shaped bacteria colonized the posterior region.

DISCUSSION

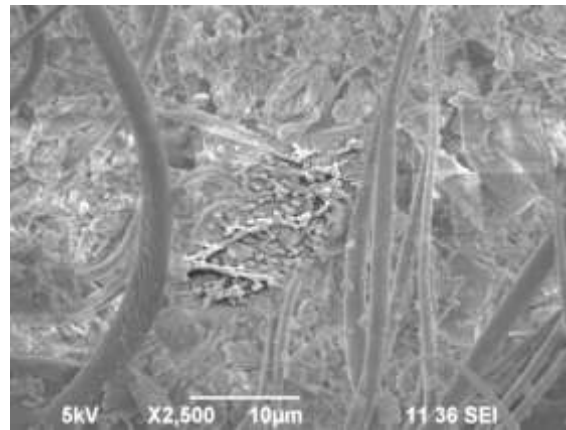
Bacteria are continually ingested with food or water. For this reason, transient microorganisms probably have a more constant and important interaction with fish gastrointestinal ecosystems as compared to terrestrial animals (Cahill, 1990). The influence of the gut flora on the host is clearly of great interest in aquaculture, particularly where poor productivity and stock losses are widespread (Ravichandran and Kannupandi, 2005). Within aquatic and other marine animals, the colonization of the digestive system by microorganisms is influenced by a number of both host-related and non host-related factors (Harris, 1993). In the gut of another freshwater culture animal, the Chinese mitten crab, investigation illustrated that Proteobacteria and Bacteroidetes might be the dominant population (Li et al., 2007).

The purpose of this study was to isolate and enumerate the bacteria flora found in the gut of fifteen mangrove crab species from the five different mangrove regions. During the study of mud crabs, lactic acid bacteria, namely *Weissella fabaria* and *Streptococcus mutans* was also identified (Li et al., 2012). These results were to promote more systematic additional knowledge on the natural intestinal bacterial communities present in crabs and enhance the understanding of the effects of aquaculture operations. Furthermore, the data will promote the development of most favourable probiotic

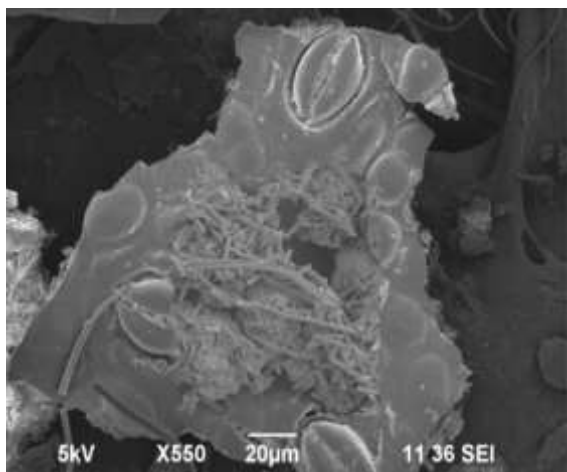
(A)



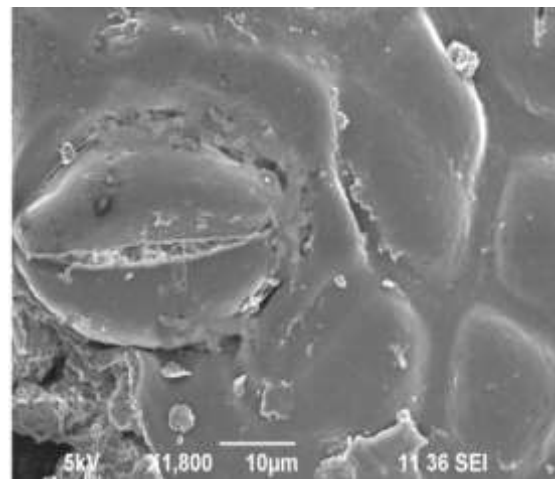
(B)



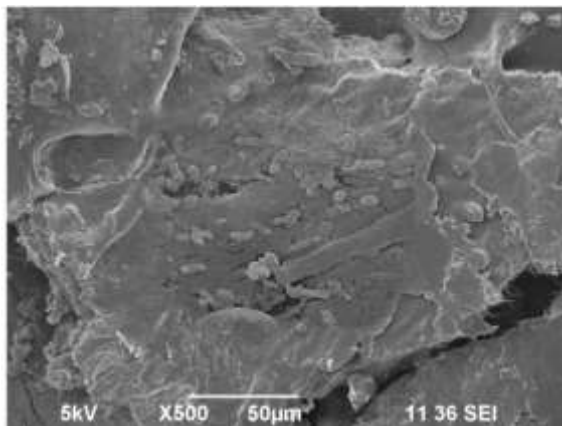
(C)



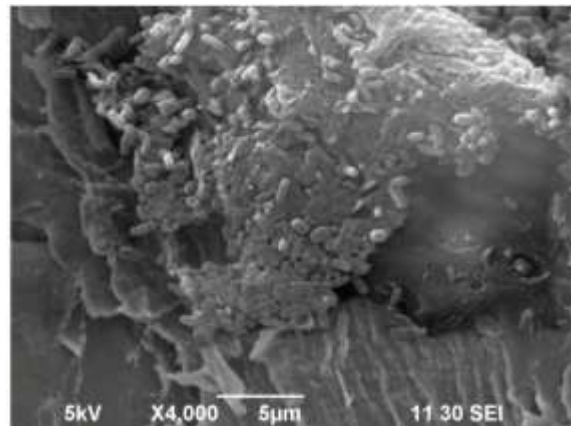
(D)



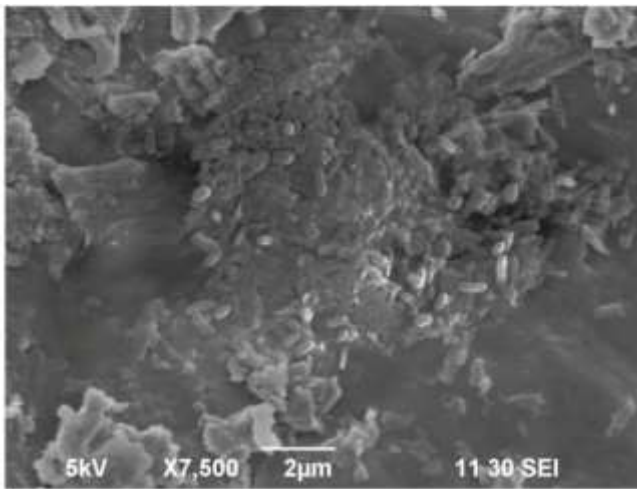
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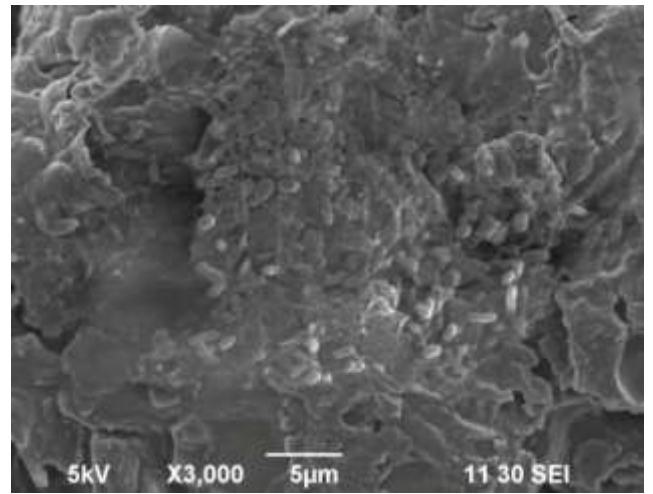
(F)



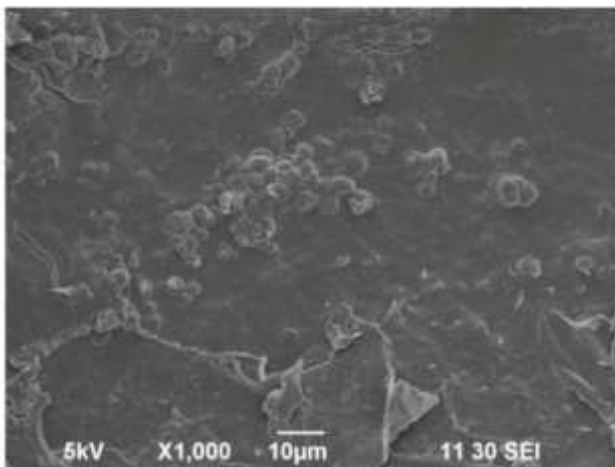
(G)



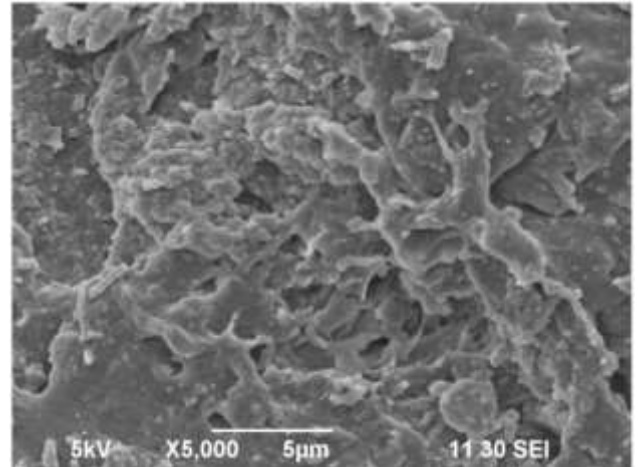
(H)



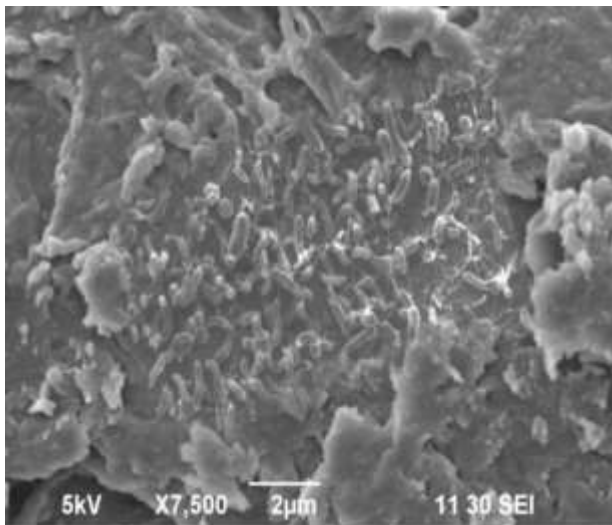
(I)



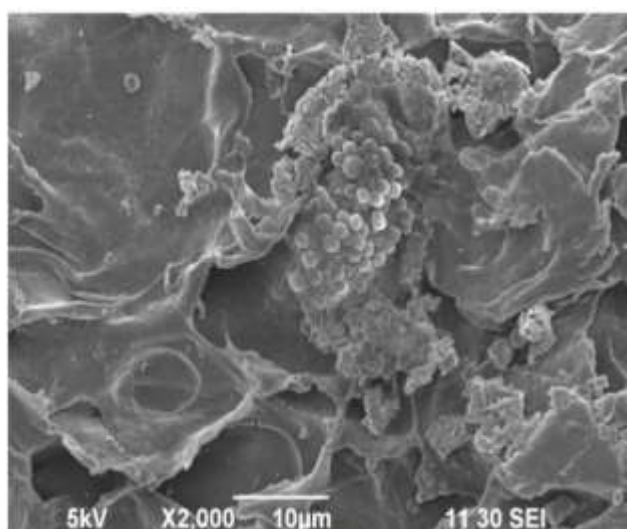
(J)



(K)



(L)



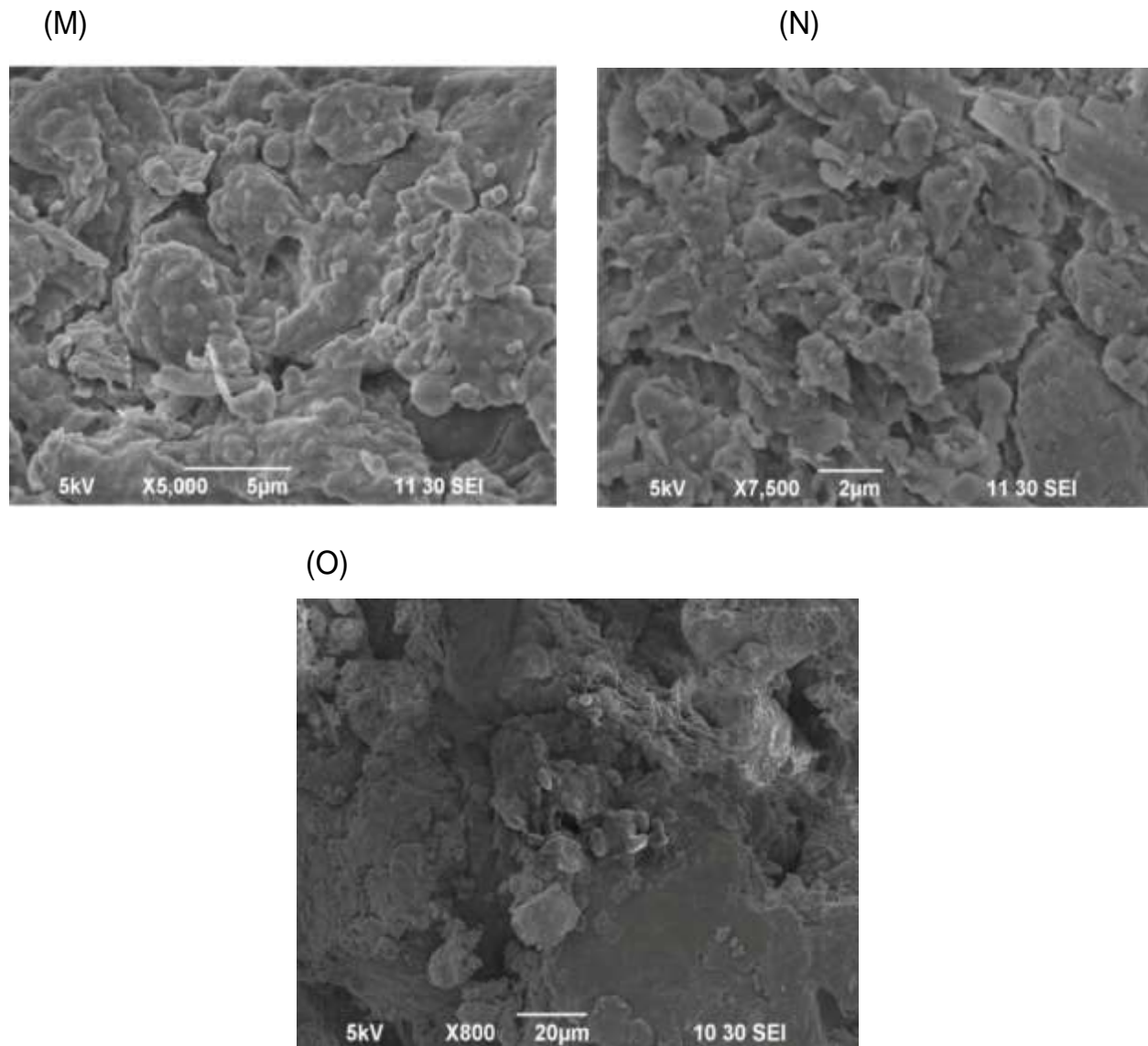


Figure 4. Scanning electron microscopic images of gut associated bacteria of various mangrove crabs. A) *Heteropanope indica*; B) *Macrophthalmus depressus*; C) *Metapograpus maculatus*; D) *Metapograpus messor*; E) *Nanosesarma batavicum*; F) *Nansesarma minutum*; G) *Neoepisesarma mederi*; H) *Neoepisesarma tetragonum*; I) *Pseudograpus intermedius*; J) *Sesarma andersoni*; K) *Sesarma bidens*; L) *Sesarma brockii*; M) *Sesarma plicatum*; N) *Uca annulipes* and O) *Uca triangularis*.

products to increase feeding efficiencies by getting better intestinal microbial balance of crabs.

The total bacterial load ranged between $0.7 \pm 0.49 \times 10^6$ and $8.9 \pm 0.13 \times 10^6$ CFU/g of crabs gut sample and it was found to be maximum ($8.9 \pm 0.13 \times 10^6$ CFU/g) in gut of *S. brockii* collected from Pichavaram mangrove region. The present investigations also support the findings of some of earlier studies. *S. brockii* were found to be the dominant species in the Pichavaram mangrove (Ravichandran et al., 2007a). Sesarmid crabs prefer

thedeaying mangrove leave for their most favourable nutritive status. Senescent mangrove leaves generally have high initial C : N ratios up to 100 which decrease in decomposing leaves. The food materials which have C : N ratios lower than 17 are considered nutritious to marine invertebrates (Russel-Hunter, 1970). The feeding activities of crabs speed up the rate of decomposition of leaf litter and may facilitate the release of nutrients to mangrove system (Lee, 1997). The mud crab, *Scylla serrata*, has a higher bacterial count in its gut than other

crabs (*Charybdis cruciata*, *Podophthalmus vigil* and *Portunus pelagicus* and *Portunus sanguinolentus*) and the luminescent bacterial floras were predominant in the hind gut and on the cuticular membranes of all the crabs (Venkateswaran et al., 1981).

The mangrove sediment harbours larger bacterial population than the water column in all the five mangrove regions. This is attributed to nutrient accumulation, precipitation of inorganic compounds and settlement of dead organic matter in the sediments (Ravikumar, 1995). Among the five stations, maximum bacterial load was noticed in the gut of crabs, sediment and water samples collected from Pichavaram. It may be due to more litter fall as compared to the other mangrove regions. Common genera in the mangrove environments were *Vibrio*, *Bacillus*, *Micrococcus*, *Pseudomonas*, *Aeromonas*, *Flavobacterium*, etc. (Sathiyamurthy et al., 1990). Similarly, in the present study, the *Bacillus* was the common and predominant genus which was present in all the examined crabs in all the stations. Several genera, *Bacteroides*, *Acinetobacter*, *Flavobacterium*, *Chryseobacterium* and *Porphyrobacter*, were identified in crab guts, and some species belonging to these genera ordinarily are related to some disease. For example, some *Bacteroides* species are opportunistic pathogens owing to their association with a variety of soft tissue and other infections (Liu et al., 2003). In shrimp, the intestinal microbial diversity investigated by molecular-dependent methods have revealed that the predominant bacterial population in the intestine of Chinese shrimp, *Fenneropenaeus chinensis* were *Proteobacteria* and *Vibrio* sp. (Liu et al., 2011). In this present study, genus *Flavobacterium* was isolated rarely in the gut of the examined crabs. Other bacterial genus, *Pseudomonas*, *Aeromonas*, *Alcaligenes*, *Photobacterium*, *Vibrio*, *Enterobacter* and *Staphylococcus* were also present in the crabs gut.

Distinct morphologies of attached bacteria are consistently associated with different crab species gut, including the pyloric fingerlets and along the anterior and posterior hindgut. There were similar reports showing that bacteria resembling that observed in this research have been reported in other marsh fiddler crabs and detritivorous thalassinid prawns (Harris et al., 1991; Harris, 1992; Pinn et al., 1999). This findings suggest that the intestinal bacterial communities in the guts of mangrove crabs still require further study. The enrichment of nutrition in the gut of mangrove crabs, and therefore, the higher inter subject variation, total diversity and abundance of the intestinal bacteria in mangrove crabs were likely the effect of these feeding methods (Li et al., 2007). Such variations could lead to developmental efficiencies and differences in digestive mechanism among the crab populations. Generally, the tropical mangrove ecosystem is endowed with a high bacterial load due to animal waste contaminations, human wastes,

the continuous shedding of foliage into the water and subsequent decomposition. Bacterial populations were high in water and sediment in the mangrove environment. Bacteria belonging to the genera *Bacillus*, *Pseudomonas* and *Aeromonas* were found at higher levels in all the different mangrove regions.

In conclusion, crabs in the various mangrove environments carry a particular bacterial flora, which reflects their environment. Moreover, some genera were identified as unique in some mangrove crabs. Additional studies are required for the use of gut associated bacteria in different aspects (that is, to degrade organic and inorganic industrial pollutants).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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