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Production of peptidic antimicrobials by bacterial populations from diverse coastal habitats of Goa, India

P G Naik & U D Muraleedharan*

Discipline of Biotechnology, School of Biological Sciences & Biotechnology, Goa University, Goa – 403 206, India

*[E-mail: usha@unigoa.ac.in]

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With conventional antibiotics rapidly losing their efficacy, marine microorganisms found in diverse salinity conditions are being explored for new antibacterial agents. Salinity is a major contributing factor to the distribution of biota in aquatic systems, and little is known about the production of antimicrobials by microbial communities in response to stressful environmental conditions. This work adds to the comprehension of bacterial abundance in estuarine and coastal beach waters of Goa and population structuring concerning their antimicrobial activity as specifically conferred by antimicrobial peptides. Total viable counts indicated higher bacterial load in estuarine water environments than in the higher salinity waters, suggesting a role of salinity variation as a driver of community composition in these habitats. In total, 82 bacterial isolates were selected, and the overall proteinaceous content in their exudates were screened for antimicrobial activity against *Salmonella typhimurium* (MTCC 91), *Staphylococcus aureus* (MTCC 3160), *Escherichia coli* (MTCC 443), *Candida albicans* (MTCC 227) and *Aeromonas hydrophila* (MTCC 1739), by the agar well diffusion method. Of these, 51.8 % showed activity against *S. typhimurium*, 30.3 % against *S. aureus*, 13.9 % against *E. coli*, 8.8 % against *C. albicans*, and 7.5 % against *A. hydrophila.* Compared with estuarine bacteria, those from more saline waters showed higher production of potent antimicrobial peptides, which were probably used to counter the high competition for resources. Such sites could, therefore, be potential niches for bioprospecting of microbes producing bacteriocin-like compounds.

[**Keywords**: Antimicrobial activity, Bacterial isolates, Peptidic antimicrobials, Salinity]

Introduction

Marine waters have distinctive properties and may harbour biota native to coastal or open ocean regions. Coastal zones are generally rich in organic matter due to terrestrial runoff and are better endowed with primary productivity. These zones include unique niches such as coral reefs, mangroves and estuarine habitats, besides rocky as well as sandy beaches[1,2]. Physico-chemical parameters such as temperature, nutrient content and salinity play an important role in determining the community composition and abundance of many organisms, which in turn defines the functional characteristics of these ecosystems. Among the abiotic factors, salinity has been extensively quoted to be the major contributor influencing the microbial community composition[3-8]. There are many reports focusing either on determining the microbial diversity in marine environments[9-11], or on the isolation of novel bioactive compounds from marine microorganisms[12]. However, only a few studies are available on bioactive metabolites obtained from different isolates in specific relation to their distinctive habitats. Due to unique habitat differences that display diversity in ambient environmental conditions (salinity, pressure, temperature, light, oxygen, pH and nutrient availability), organisms tend to develop various chemo-physiological processes for survival. Such metabolic processes of marine microbes encourage the synthesis of a range of bioactive molecules exhibiting activities such as antimicrobial, anticancer, antiviral and antifungal[13,14]. While their isolation was initially carried out from sites at random, there is now a growing recognition that the sampling location could play an important role in increasing the success rate of bioactivity discoveries[15,16].

Marine microbes are known to produce various classes of metabolites such as polyketides, terpenes, peptides, and alkaloids which have been shown to exhibit antimicrobial and antiviral activity[17,18]. As the conventional small molecules lose their potency quickly due to antimicrobial resistance[19], one important class which has been drawing increasing attention is that of proteinaceous or peptidic antimicrobials from bacteria, also known as bacteriocins. It has the advantage of being minimally

vulnerable to such resistance. Killing mechanisms adopted by bacteriocins are (a) formation of ion-permeable pores in the cytoplasmic membrane, (b) degradation of cellular DNA, and (c) alteration in the structure of the 30S RNA subunit with subsequent degradation of rRNA or tRNA. These peptides have different structural diversity and properties and vary significantly in molecular weight, from small peptides (microcins, < 10 kDa) to large ones (colicins, > 80 kDa)[20]. Bacteriocins are majorly produced by Gram-positive bacteria and are divided into four classes. Class I includes lantibiotics, which are hydrophobic, thermostable, and pore-forming peptides with a low molecular weight (< 5 kDa). Class II peptides are also heat-stable and small in size (< 10 kDa) and mainly induce destabilisation and permeabilisation of the bacterial membranes or cause pore formation into the membrane. Class III bacteriocins are large peptides (> 30 kDa) and may be heat-labile, lytic or non-lytic. These bacteriocins have an antibacterial activity linked to enzymatic activity (such as endopeptidase), leading to the disruption of the bacterial cell wall. Class IV bacteriocins contain lipid or carbohydrate parts which disrupt the bacterial cell membrane. Due to their peculiar structure these peptides are sensitive to several enzymes (such as glycolytic or lipolytic enzymes)[21]. Bacteriocins produced by Gram-negative bacteria are divided into four major classes: colicins (10 kDa), Colicin-Like Bacteriocins (CLB), Phage Tail-Like Bacteriocins (PTLB), and microcins[22].

Microbes thriving under varying salinity conditions can be explored further to obtain natural products. A large number of the strains known to produce protein/peptide antibiotics against the most common pathogenic bacteria have been isolated from various marine environments. Bacitracin, which showed inhibitory activity against many Gram-positive organisms, is produced from a marine bacterium *Bacillus licheniformis*[23]. Enterocin isolated from *Enterococcus faecium* found in the mangrove environment of the Vellar estuary on the east coast of India showed antimicrobial activity against a broad range of Gram-positive and Gram-negative pathogens *viz.*, *S. aureus*, *B. subtilis*, *E. coli* and *Vibrio* sp.[24]. Pumiviticin, a bacteriocin derived from the marine bacterium *Bacillus pumilus* DR2 isolated from seawater samples from Ennore (Tamil Nadu, India), showed antimicrobial activity against *Lactobacillus jugurti*, *Lactobacillus casei*, *Lactococcus lactis*, *Leuconostoc mesenteroides* and *Listeria monocytogenes*[25]. In addition to clinical applications, such peptides have a wide variety of applications such as in the food industry, animal husbandry, agriculture, aquaculture and are also used to tackle biofilm and biofouling challenges[26,27].

To the best of our knowledge, this is the first report on a possible role of the ambient salinity in antimicrobial peptide production potential of bacteria sourced from varying salinity zones such as Divar mangrove area (fluctuating salinity zone), Miramar and Cacra beaches (less saline, near-estuarine regions) as well as Vagator and Anjuna beaches (coastal waters) of Goa, India. While some bacteria isolated from salinity zones have been reported to show antibiotic activities, the present work focuses majorly on proteinaceous antimicrobials (bacteriocins), as distinct from antibiotics as a class.

Materials and Methods

Sites and sample collection

The coastline of Goa, India, is characterised by continuous stretches of sandy beaches, with several places intersected by estuarine river systems that empty into the Arabian Sea. For the isolation of marine bacteria, five of Goa's geographical areas of study were sampled (Fig. 1). The sites were selected for their salinity characteristics. The samples were collected in the month of January 2018 from estuarine waters near the Divar Mangrove (DM) area, which is separated from the mainland by the Mandovi estuary; Miramar beach (ME) intertidal zones; the near-estuarine regions at Cacra (CE) beach; and the more typically saline waters of Vagator (VC) and Anjuna (AC) beaches which had minimal influence from river water discharge. Surface water sample from each site

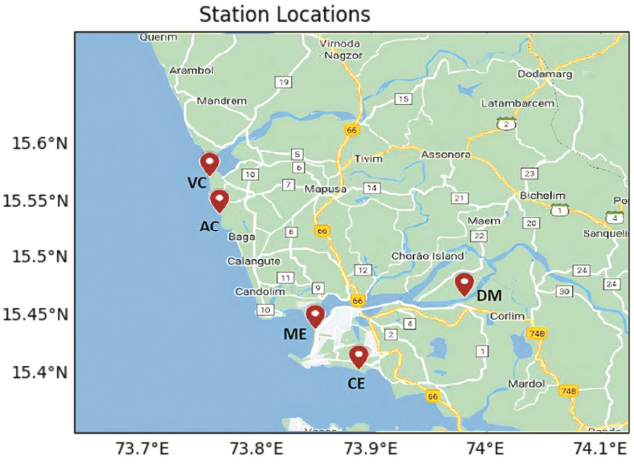

Fig. 1 — Geographical location of study area and sampling sites: DM - Divar mangrove area, ME - Miramar beach, CE - Cacra beach, VC - Vagator beach, and AC - Anjuna beach

(approx. 1 L) was collected in pre-sterilised screw-capped bottles, transferred on ice to the laboratory and processed within 24 h.

Measurement of physico-chemical parameters at sampling sites

Salient parameters of the water samples, such as temperature, salinity, conductivity, Total Dissolved Solids (TDS) and Dissolved Oxygen (DO), were determined on the field using a multiparameter instrument (CyberScan PC 650, Eutech Instruments, Thermo Fisher Scientific, India) and pH was measured on a digital pH meter (Eutech pH meter-700, Thermo Fisher Scientific, India).

Isolation and enumeration of bacteria

The bacterial population in water samples was enumerated using the standard spread-plate technique[28,29]. Water sample (1 mL) collected from each location was serially diluted using sterile saline and 0.1 mL spread-plated on Zobell Marine Agar (ZMA), Nutrient Agar (NA) and Seawater Agar plates (SWA) which were then incubated at room temperature for 24 h, 48 h and 72 h, respectively, for enumeration of Total Viable (culturable) bacterial Counts (TVC). The ZMA and NA were procured from HiMedia, while, for the SWA preparation, 3 g agar powder (HiMedia) was added to 100 mL aged and filtered seawater and autoclaved.

Test pathogens used for screening of antagonistic activity

Pure cultures of *Salmonella typhimurium* (MTCC 98), *Staphylococcus aureus* (MTCC 3160), *Escherichia coli* (MTCC 443), *Candida albicans* (MTCC 227) and *Aeromonas hydrophila* (MTCC 1739) were used for the study. These bacterial and fungal strains obtained from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India were maintained on nutrient agar, prior to use.

Antimicrobial assay

Antimicrobial activity of the isolates was evaluated by the agar-well diffusion method, as per Shanmugaraju *et al.*[30]. The predominant isolates were selected based on morphological characteristics, subcultured and maintained on ZMA. The test pathogen inocula, prepared by transferring a loopful of culture into 100 mL nutrient broth and incubating for 24 h on an orbital shaker, were swabbed onto NA plates. The isolates were allowed to grow in Zobell marine broth for 24 h on a rotary shaker. The broth culture was then centrifuged at 6,000 rpm for 10 min at 4 °C to obtain the Cell-Free Supernatant (CFS). Solid ammonium sulphate was added to 90 % (w/v) saturation, and the CFS was stirred for 3 to 4 h at 4 °C to maximally precipitate proteinaceous molecules. Centrifugation was then carried out at 10,000 rpm for 30 min and 4 °C. The pellet obtained was re-suspended in 10 mM sodium acetate buffer (pH 5.6). Wells (6 mm) were punched onto the test pathogen NA plates using a borer, and 30 µl of CFS (crude or partially purified) was added into the wells. Zones of inhibition were measured after incubation for 24 h at 37 °C.

Protein estimation

Ammonium sulphate precipitated samples suspended in 10 mM sodium acetate buffer, pH 5.6 were analysed for protein content by the Bradford method[31], using Bovine Serum Albumin (BSA) as standard.

Results and Discussion

The physico-chemical parameters recorded at each sampling site are summarised in Table 1. The pH value, which represents the degree of ionisation of the sampled water, ranged from 7.36 to 8.5. The temperature of the surface water at these sites varied from 28 to 29.8 °C. Salinity variations were from 26.9 to 33.4 psu; Vagator (VC) and Anjuna (AC) beach sites exhibited the highest salinity (33.04 to 33.42 psu), as expected. The Miramar (ME) and Cacra (CE) sites were comparatively less saline, being influenced by the input of freshwater from the Mandovi and Zuari rivers. Salinity of estuarine waters around the Divar Mangrove area (DM) was 26.9 psu. Notwithstanding interannual variabilities and

Table 1 — Variations of ambient parameters in the surface water samples collected from different sampling locations: DM - Divar mangrove area, ME - Miramar beach, CE - Cacra beach, VC - Vagator beach, and AC - Anjuna beach

Location (& Station code)	pH	Temperature (°C)	DO (mg/L)	Salinity (psu)	Conductivity (ms)	TDS (ppt)
Divar (DM)	7.36±0.07	28±0	6.81±0.37	26.95±0.18	43.35±0.93	28.86±2.81
Miramar beach (ME)	8.31±0	29.8±0	6.01±0	31.96±0.15	48.95±0.21	34.29±0.26
Cacra beach (CE)	8.35±0.01	28±0	5.97±0.32	32.14±0.08	49.18±0.12	34.44±0.08
Vagator beach (VC)	8.41±0.01	29.8±0	6.68±0.11	33.04±0.21	50.42±0.28	35.31±0.2
Anjuna beach (AC)	8.50±0	29.8±0	6.70±0.12	33.42±0.18	50.93±0.25	35.63±0.12

seasonal influences, these values are in agreement with the data from earlier reports[32,33], where salinity values ranging from 31 to 35 psu were reported in coastal beach waters and from 24 to 27 psu in mangrove areas of Goa. In the present study, Miramar and Cacra beach areas were categorised as 'near-estuarine' regions and the mangrove habitat as 'estuarine' environment, while the Vagator and Anjuna sites better conformed to 'coastal ocean' environments. All these typically exemplify the dynamic environmental conditions that characterise the estuarine and marine waters around Goa.

Dissolved oxygen (DO), being one of the limiting factors for the growth and functioning of marine microorganisms, becomes a very useful parameter to gauge water quality. The observed DO values ranged between 5.97 and 6.7 mg/L. Total Dissolved Solids (TDS) comprise of inorganic salts (bicarbonates, chlorides and sulphates of calcium, potassium, magnesium and sodium) and some amount of organic matter. The measured TDS values ranged from 28.8 to 35.6 ppt. Electrical conductivity depends upon the quantity of dissolved salts in the water, and it ranged from 43.3 to 50.9 ms. Maximum and minimum values for pH, DO, salinity, conductivity and TDS were recorded at AC and DM sites, respectively, in the month of January.

Although, in general, abiotic parameters such as salinity, nutrient concentration and organic matter would all influence the composition of natural microbiota[34,35], salinity, in particular, influences the osmoregulatory functions of organisms and would therefore be a dominant factor in shaping microbial diversity and population in the marine waters[36]. Previous studies have shown the influence of salinity on microbial community composition[37]. There are, however, very few reports on bacterial abundance over a range of salinities especially in relation to their bioactivities. The focus of this study was hence directed to evaluate the influence of salinity ranges of estuarine and marine habitats on the total viable bacterial count and the production of peptidic antimicrobials.

Total viable count

Bacterial abundance in the estuarine and coastal waters of Goa was gauged by the TVC, which would provide an estimate of their culturable load in these waters (Fig. 2). The values were highest at location DM, as 660×10^3 cfu/mL and 210×10^2 cfu/mL,

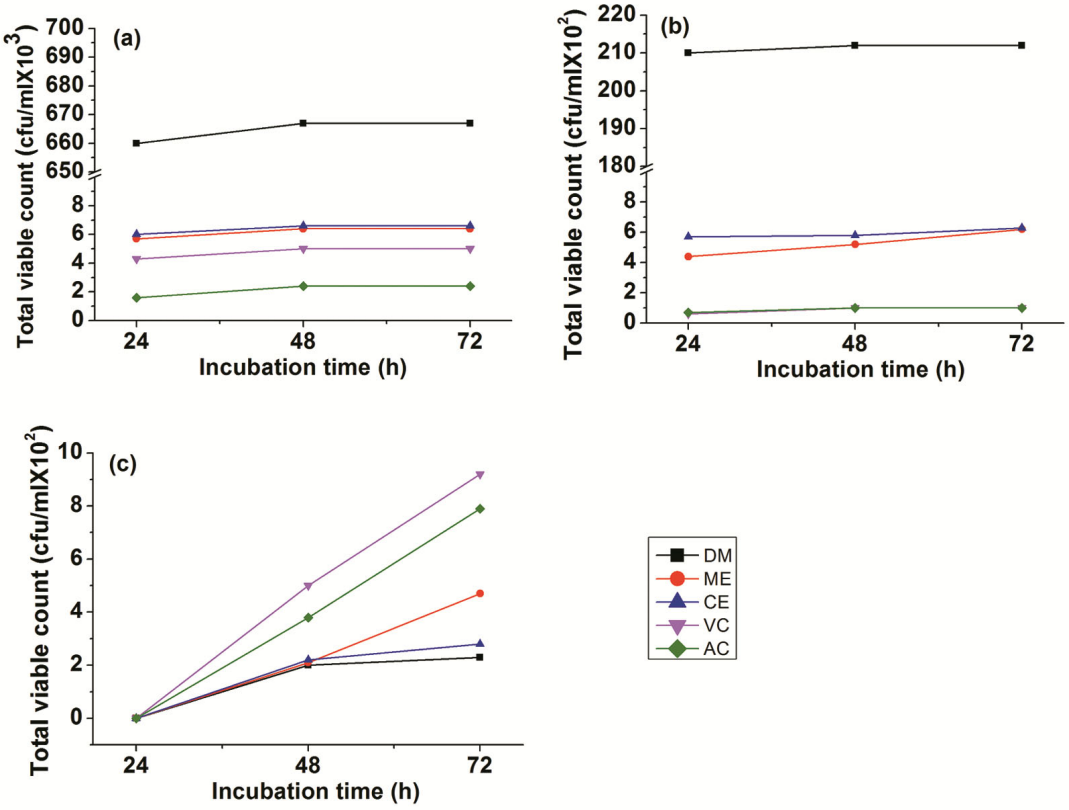

Fig. 2 — Total viable count at the sampling locations of various salinities on (a) Zobell marine agar, (b) Nutrient agar, and (c) Natural seawater agar. DM - Divar mangrove area; ME - Miramar beach; CE - Cacra beach; VC - Vagator beach; and AC - Anjuna beach

respectively, upon incubation on ZMA and NA media for 24 h. In all cases, only a slight increase in TVC was observed after 48 h of incubation, which remained unchanged even at 72 h, indicating that the exponential phase of growth was complete even at 24 h of incubation. No colonies were observed on SWA at 24 h, and even after 48 h of incubation, the highest TVC recorded was only 5×10^2 cfu/mL at VC. Although a continued increase to 9.2×10^2 cfu/mL was recorded in SWA medium at 72 h, this was markedly lower than the values in richer media such as ZMA and NA. This slow growth may be attributed to autoclaving of the medium, which could have affected the natural nutrient availability, as against filtration, which might have been a better choice.

The more significant values of TVC as recorded for growth in ZMA and NA media at 48 h were assessed in relation to ambient salinity at the sampling sites. It was observed that the values were higher in samples from a lower salinity environment (mangrove area) and decreased with increasing ambient salinity (Fig. 2a & b). Other parameters at the sampling sites besides salinity might also have contributed to the TVC differences, considering the trend of variations of pH, temperature, conductivity and TDS (Table 1).

A total of 82 distinct bacterial isolates, as obtained on three different media, were selected based on differences in colony morphology and Gram character. Estuarine samples showed more diversity of colonies than marine samples. The majority of isolates (71 – 77 %) obtained from the five sampling sites were Gram-negative (Fig. 3b, hatched bars, *cf.* Fig. 3a, hatched bars), in concurrence with a recent article that cites Gram-positive bacteria contributing to 14 % of the total bacterial count in seawater[38].

Among the Gram-positive bacteria, a higher percentage of isolates obtained from estuarine and coastal waters were antimicrobially active (solid bars, Fig. 3a), while among the Gram-negative isolates, those exuding antimicrobially active compounds were predominant in the near-estuarine regions (solid bars, Fig. 3b). Unlike those introduced by runoff from terrestrial ecosystems, indigenous producers of antimicrobial peptides from the marine environment were mainly strains of Gram-positive marine bacteria, despite the relative abundance of Gram-negative species[39]. Although Gram-positive bacteria are basically abundant in soils and could get introduced into near-shore marine environments, their survival rates are low in competition with the indigenous

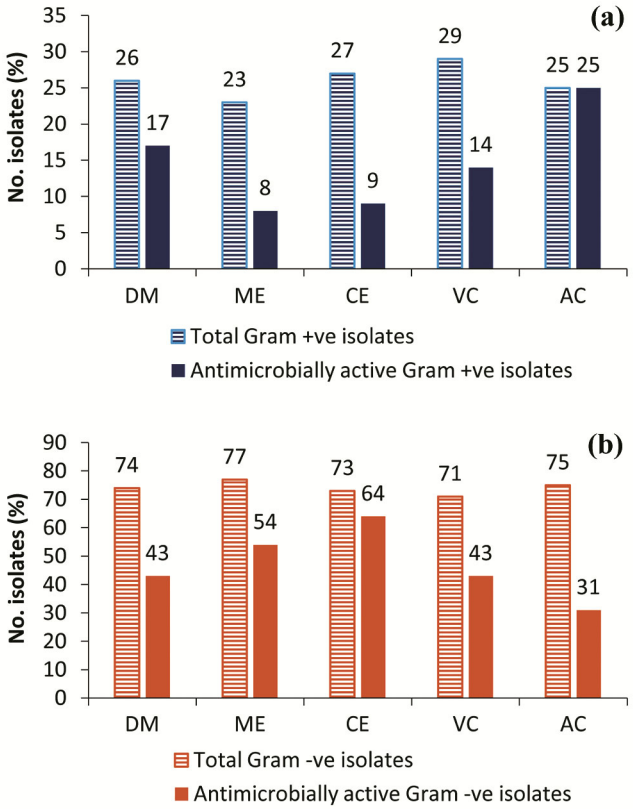

Fig. 3 — Percentage of (a) Gram-positive, and (b) Gram-negative isolates amongst the culturable isolates obtained during the sampling. The solid bars represent the relative percentage of Gram-positive and Gram-negative isolates that showed antimicrobial activity. The respective percent values (rounded up) are indicated above each bar

better-adapted Gram-negative species[40]. Besides, on account of their membrane porosity characteristics, Gram-positive organisms would be more sensitive to deleterious compounds in the vicinity. Such compounds that may be effectively transported across the thick peptidoglycan layer of Gram-positive bacteria lack, in many cases, the appropriate chemical properties to cross the vastly different glycolipid layer and membranes characteristic of Gram-negative bacteria[41,42].

Antimicrobial activity in proteinaceous molecules

The search for proteinaceous or peptide antimicrobials from marine microbes as an alternative to conventional antibiotics being the focus of the current study, bioprospecting for antimicrobial activity was performed using the well diffusion assay (Fig. 4). Partial purification by ammonium sulphate precipitation would serve to extract proteinaceous material more specifically, besides also concentrating such active molecules. It is interesting to note that

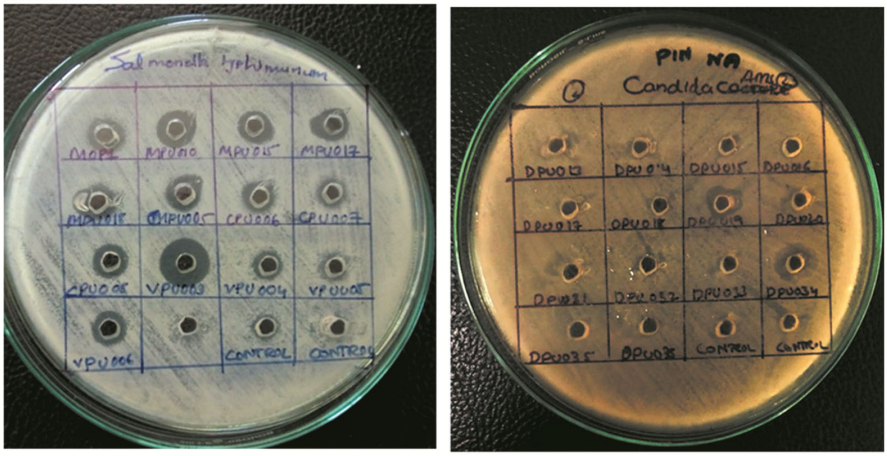

Fig. 4 — Antimicrobial susceptibility testing using the well diffusion method

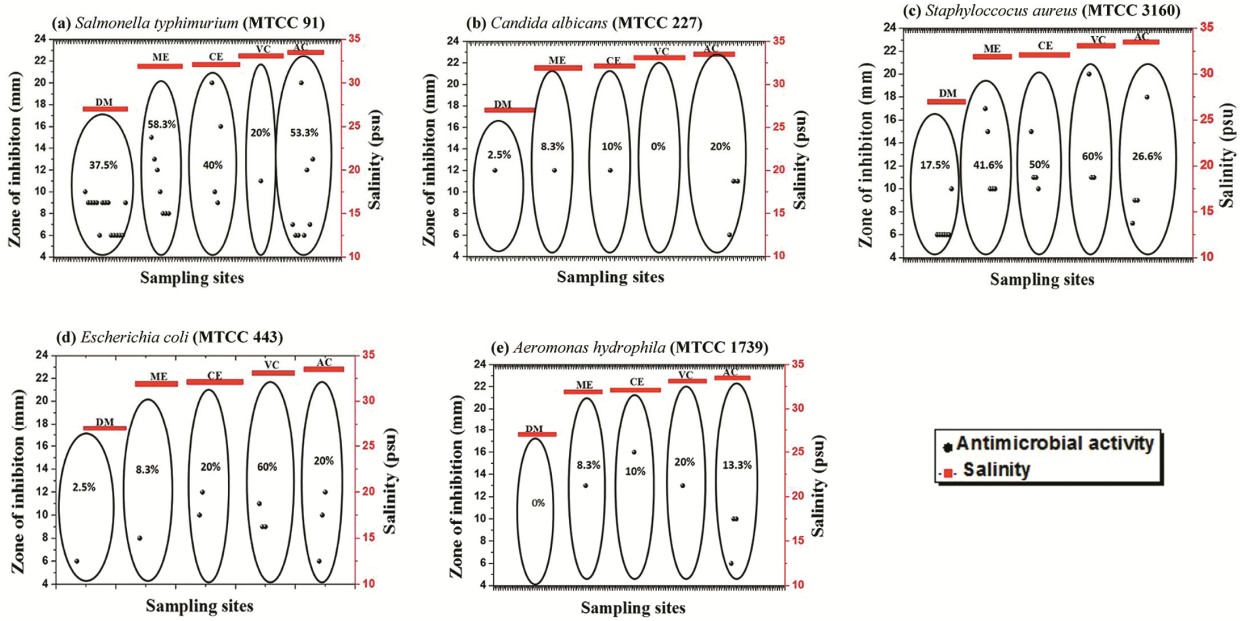

Fig. 5 — Antimicrobial activity in partially purified exudates of the isolates as against five pathogenic test cultures [(a) *Salmonella typhimurium* (MTCC 91), (b) *Candida albicans* (MTCC 227), (c) *Staphylococcus aureus* (MTCC 3160), (d) *Escherichia coli* (MTCC 443), and (e) *Aeromonas hydrophila* (MTCC 1739)], and viewed in relation to sampling site salinity variations

taken as a whole, about 61 % of the culturable isolates had antimicrobial activity associated with proteinaceous material (data not shown). The isolates displayed broad-spectrum inhibitory effects on the five test pathogens used, and the antimicrobial activity in partially purified CFS samples was higher than in the crude CFS preparations. The significantly enhanced activity upon partial purification is not surprising, considering that antimicrobial peptides and proteins are generally not generated in high amounts by the producer strain[43]. In fact, the proteinaceous content in these active preparations ranged from 0.7 to 2 mg/mL (data not shown).

Salinity variations and antimicrobial activity

As detailed above, a marked difference was observed in the microbial load from estuarine to coastal marine ecosystems and in their production of antimicrobial peptides/proteins. The comparative analysis using the partially purified proteinaceous samples is presented in Figure 5, in relation to the different sampling sites and test pathogens used. In the estuarine environment, high bacterial abundance contrasting with low antimicrobial activity was observed against all five pathogens tested. A possible explanation could be that many marine and freshwater microflora that entered into the estuarine mangrove

environment (mixing zone) were no longer active because of the rapid salinity fluctuations encountered. In contrast, the seawater salinity tolerant microbes appear to be a better-adapted community and are metabolically active[44]. Results of the present study indicated that as compared to estuarine bacteria, those in other areas showed the production of more potent antimicrobial peptides probably used to counter the high competition for resources. Hence, these could be a more promising source for bacteriocin-like compounds.

Conclusion

Eighty two bacterial isolates were screened from distinct coastal environs of Goa. Bacterial abundance, antimicrobial potential of the isolates and their structuring in relation to the ambient salinity variations has been specifically investigated. There was a progressive increase in TVC from estuarine to higher salinity marine waters. Isolates from more saline coastal regions had stronger antimicrobial potential than those from the estuarine habitats. This bioactivity could be largely attributed to peptidic molecules. The present study adds to the knowledge of distribution of marine bacteria with antimicrobial potential for furthering biotechnological applications to address and alleviate rising concerns about antibiotic resistance. Further work on the isolation and characterisation of such antimicrobial peptides would open up avenues in the search for novel antimicrobial peptides and thereby, more such molecules would continue to enter clinical trials. Also, novel compounds with different chemical structures and biological activities would become available to counter the ever-growing antimicrobial resistance.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

PGN: Laboratory and field investigations, formal analysis, use of software and writing original draft of the manuscript; and UDM: Conceptualisation, supervision, and review & editing of the manuscript.

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