

Antimicrobial activity of coral-associated beneficial bacteria against coral disease causing microbial pathogens

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Abstract

Microbial infection of immune-compromised corals influences disease severity, resulting in coral mortality. However, coral-associated beneficial bacteria are known to produce antimicrobial compounds that prevent the growth of potential pathogens and invading microbes. Hence, beneficial bacteria associated with coral *Porites lutea* were isolated and antimicrobial protein and bioactive secondary metabolites were extracted and tested for their antimicrobial activity against putative prokaryotic and eukaryotic coral pathogens. Bioactive secondary metabolites exhibited remarkable antagonism against various coral pathogens such as *Serratia marcescens*, *Vibrio* species and *Aspergillus sydowii*. Besides, the metabolites of *Cobetia marina*, *Cobetia amphilecti*, *Pseudoalteromonas neustonica* and *Virgibacillus halodenitrificans* manifested notable inhibition against the protozoan ciliates (*Uronema marinum*, *Holosticha diademata*, *Cohnilembus verminus* and *Euplotes vannus*) and zooplankton that are known to be involved in the secondary pathogenesis in coral diseased lesion progression. Thus, the present study may benefit in understanding coral-associated beneficial bacteria for their antagonistic interactions with microbial pathogens, as well as their potential involvement in reducing coral disease severity.

Keywords : ciliates, corals, disease, infection, antagonism, secondary metabolites.

Abbreviation: BMCs, Beneficial microbes of corals.

1 Introduction

Coral reefs constitute a highly diverse and valuable ecosystem on the planet [1,2]. Scleractinian corals are the basis for highly diverse coral reef ecosystems [3-7]. They hold a massive, spectacular limestone structure that exhibits high productivity, beauty and biological diversity [8]. Their shallow water reef ecosystems often build a systemic framework that supports a vast micro and macro biological diversity [9,10]. Several thousands of microbial species are harboured (bacterial cells 10^7) in each square centimetre of these coral surfaces which is ten times greater than the surrounding seawater [11-13]. In recent decades, coral reefs have been identified as the most fragile marine ecosystem, with disease outbreaks posing a severe threat to one of the planet's most diversified ecosystems [14-16]. Increasing disease prevalence had led to coral reef decline and subsequent coral coverage losses [16-18].

Microorganisms, on the other hand, serve a critical role in sustaining the function and health of this multicellular life and the habitat in which it thrives [19]. Thus, the Coral-associated microbes such as bacteria, Archaea, fungus, dinoflagellates, phage and eukaryotic viruses play a vital role in the biogeochemical cycling of material transformation and the maintenance of coral reef ecosystem health [20-23]. Many bacteria associated with corals hold potentially significant host functions in sulfur cycling and nitrogen fixation that benefits coral to succeed in oligotrophic waters. Some protect the coral host from pathogens by potentially interrupting the cell-to-cell communication and antimicrobial compound production [24-29]. Thus, beneficial microbes of corals (BMCs) enhance coral health by symbiotic association with a host coral that includes holobiont nutrient cycling, and exclusion/antagonism of pathogens [30-33]. Therefore, the coral reef ecosystem is regarded as an untapped resource with enormous biotechnological potential [34,35].

High diversity of marine microbes associated with corals, sponges and algae are widely established to be potent biological active molecule producers, representing valuable sources for novel compounds [36-38]. Around three fourth of bioactive products discovered in oceans are from marine invertebrate-associated bacteria [37]. These microorganisms gain their ability to produce secondary metabolites by the non-ribosomal protein synthetase and polyketide synthase gene clusters that express a diverse group of compounds with enormous therapeutic potential [39,40]. Several attempts have been undertaken to test the synthesis of antibacterial substances by coral-associated microbial isolates [33, 41-44]. Coral *Oculina patagonica* mucus isolated bacteria had exhibited antagonism against coral pathogens *Vibrio coralliilyticus*, *V. shiloi* and *Serratia marcescens* [43]. Similarly, many corals-associated bacterial isolates displayed antagonistic activity towards the putative coral pathogens [33, 44]. Thus, exploring coral symbiotic microorganisms in search of novel active chemicals is beneficial

and valuable. Therefore, current study aimed to examine the culturable bacterial diversity of diseased and healthy tissues of the scleractinian coral *Porites lutea* for putative beneficial microbes and characterize their antimicrobial substances against bacterial, fungal, and protozoan coral pathogens.

2 Materials And Methods

2.1 Coral sample collection and processing

Coral samples such as water, tissue and sediment were collected from the healthy, diseased and algal-dominated corals *Porites lutea* in June 2018 and February 2019 from the Gulf of Mannar (Fig.1) of Indian Ocean (Latitude: N 09°17' 36.2", Longitude: E 079°08'12.6"), India. The collected samples were transported to the laboratory and subjected to isolation of culturable microbes. Samples (water, tissue and sediment) were spread over Zobell Marine Agar (HiMedia) with a serial dilution ratio of 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} . The inoculated plates were then incubated at $28 \pm 2^\circ\text{C}$. Morphologically distinct colonies were streaked onto the same isolated media and thus obtained pure cultures were stored at 4°C and as glycerol stock (-80°C).

2.2 Bacterial strains and culture conditions

The marine originated strains were routinely grown at 28°C in Marine Broth (Himedia-M385) and well diffusion assay was performed in Mueller Hinton Agar (Himedia-GM1084). The secondary metabolites were extracted using International Streptomyces Project 2 (ISP2) (Yeast extract 4g/l, Malt extract 10g/l, Dextrose 4g/l, Agar 2% and NaCl 2%) broth. The pathogenic stains were procured from CSIR-IMTECH (MTCC), Chandigarh, India and their designations are *Vibrio vulnificus* (MTCC-1145), *V. parahaemolyticus* (MTCC-415), *V. harveyi* (MTCC-7954), *Staphylococcus aureus* (MTCC-3160), *Serratia marcescens* (MTCC-10436), *Aspergillus sydowii* (MTCC-10126) and maintained as per MTCC instructions. MRSA (Methicillin resistant *Staphylococcus aureus*), *Candida albicans*, *Escherchia coli*, and *Chaetomium globosum* are isolated and maintained in the laboratory. Few other *Vibrio* sp. strains of six were isolated from coral diseased tissues using TCBS media were utilized for cross streak assays.

Individual colonies were introduced to the same isolation broth and cultured for molecular characterization of the isolated pure cultures. The genomic DNA of the culture pellet obtained after centrifugation (8000 rpm 20 mins) was extracted using the phenol- chloroform method. PCR amplification of the 16S rRNA gene was done with the bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR-amplified samples were purified by polyethylene glycol (PEG 6000). Sequencing was done with 3130xl Genetic Analyzer (Applied Biosystems), CSIR- National Institute of Oceanography, Goa. The

obtained 16S rRNA gene sequences were searched in National Centre for Biotechnology Information (NCBI) BLASTn and EzTaxon and the closely related species were identified. Thus obtained nucleotide sequences were deposited in the GenBank database under the accession numbers of *Bacillus firmus* (OQ834639), *B. flexus* (OQ834643), *B. oceanisediminis* (OQ834662), *B. paramycooides* (OQ834605), *Cobetia amphilecti* (strain 4) (OQ834663), *C. amphilecti* (strain 6) (Q834664), *C. marina* (strain 1) (OQ834633), *C. marina* (strain 2) (OQ834607), *Pseudoalteromonas carrageenovora* (OQ834603), *P. espijana* (OQ834612), *P. neustonica* (OQ834627), *Virgibacillus halodenitrificans* (OQ834595), *Psychrobacter pulmonis* (OQ834597), *P. alimentarius* (OQ834613), *Serratia marcescens* (OQ834630), *S. nematodiphila* (OQ834629).

2.3 Screening of potent Beneficial microbial culture

The bacterial species identified by 16S rRNA molecular characterization were screened for their host beneficial and harmful nature with the help of previously published literature. Besides, antimicrobial cross or perpendicular inhibition tests were performed to examine the antipathogenic capabilities of isolated beneficial microorganisms against the isolated pathogens. Briefly, each beneficial isolate was streaked as a middle line of a single streak in ISP2 (2% NaCl) media and incubated at 28 ± 2 °C until visible growth appeared. The test pathogens of 10 µl were then streaked perpendicular to the middle line of the streak, and they were then left to incubate at 28 °C overnight. The obtained inhibitory zone was measured from the edge of the middle line streak to the resulting pathogen colony.

2.4 Extraction and screening of antimicrobial protein of beneficial bacteria

The potent coral-beneficial bacteria studied by cross inhibition were chosen for antimicrobial protein extraction. Briefly, beneficial isolates were cultured in marine broth and centrifuged (8000 rpm, 10 mins). The extracellular and intracellular proteins were extracted from the culture supernatant and pellet respectively. Extracellular protein was extracted by ammonium precipitation (52.15 g in 100 ml), and the intracellular protein was extracted briefly by Acetone precipitation. The culture cell pellet was washed twice with PBS (pH 7.4) by centrifugation (8000 rpm 10mins) and soaked in ice-cold acetone for 30 mins, followed by a wash in 10% SDS. Then the centrifuged pellet was suspended in minimum volumes of PBS and frozen (-20 °C) for further analysis. Later both the extracted proteins (100 µl) were checked for antagonism against the isolated coral pathogens by well diffusion assay on Muller Hinton agar (MHA, Himedia). PBS was taken in the place of control and the inhibition zones formed against the pathogens were measured and recorded.

2.5 Extraction and screening of antimicrobial secondary metabolite of beneficial bacteria

The coral-beneficial bacteria screened by cross-inhibition assay were cultivated in ISP2 broth (pH 7.2) for 24 h at 28 °C and their cells were harvested by centrifugation (8000 rpm, 10 mins). The secondary metabolites were extracted from the cell-free supernatant with equal amounts of ethyl acetate in the separating funnel. Simultaneously the cell pellet was sonicated (Probe Sonicator PRO 650- LABMAN) with methanol: dichloromethane (1:1) and their organic layer was collected by centrifugation. The crude extracts of cell pellets and supernatant were concentrated under a vacuum (Roteva- Rotary evaporator) and suspended in minimum amounts of methanol. Then the anti-pathogenic activity of both the intracellular and extracellular metabolites (25 µl) was checked on Muller Hinton agar (MHA, Himedia) by well diffusion method. Inhibition zones were measured and noted.

2.6 Activity of secondary metabolite antagonism in bacterial broth culture

The antagonism of secondary metabolites against the coral pathogens, cultured in liquid broths was checked to ascertain the antimicrobial activity attained in solid agar medium. Various concentrations of metabolites were introduced against a known concentration of bacterial pathogen. After a period of incubation, their cell number was calculated from the optical density measured. To the sterile 1 ml (made upto) ZMB, different concentrations such as 25, 50, 75 and 100µl of metabolites were added, to which 5% (approx. 0.7 OD) of o/n grown pathogenic microbes were introduced and incubated under suitable conditions. Controls were maintained without metabolites and with the solvents (0.5 ml Methanol+ 1.5 ml 5% DMSO) that are used to dissolve the metabolite. After 12 hours of incubation, the bacterial cell density was measured (600 nm) in a spectrophotometer. The values were recorded and graphs are plotted accordingly.

2.7 Protozoan ciliate inhibition

The protozoan pathogens of corals that were already isolated and characterized earlier from our laboratory [45] were subjected to an antagonism study against the secondary metabolites of coral-beneficial bacteria. This was achieved through the ciliate motility test. The motility of the ciliate in the presence of varied metabolite volumes towards the yeast agar block was calculated as the percentage of motile ciliates. Four protozoan ciliates such as *Holosticha diademata*, *Uronema marinum*, *Cohnilembus verminus* and *Euplotes vannus* were taken up for the study. Around 500 numbers of each ciliate species were taken from the already maintained cultures. Then 10, 20, 30 and 40 µl of metabolite samples were loaded to 2 ml (made upto) of Sea water containing 100 µl of ciliate culture (500 nos) in each well (microtitre well plate) and incubated for 16 mins. After incubation, the total number of motile

ciliates was counted. The methanol controls were compared with the treated and likewise all the experiments were done in triplicates. After incubation the number of motile ciliates was counted and their percentage of motility was recorded.

2.8 Zooplankton inhibition

The coral-associated zooplanktons were collected beneath the coral dead tissues that were maintained in the laboratory for acclimation. The collected zooplanktons of about 30 numbers were aliquoted in microtiter plates of 12 wells and each well was added 2 ml of seawater (final volume). The metabolites of *Cobetia amphilecti* 6, *C. amphilecti* 4, *Cobetia marina* 1, *C. marina* 2, *Virgibacillus halodenitrificans*, *Pseudoalteromonas neustonica* and *Bacillus flexus* (CA6, CA4, CM1, CM2, VG, PN and B.fl) was added in subsequent volumes (40, 50, 60 and 70 µl) and incubated for 30 mins. The positive and negative controls were compared with the experiment. All experiments were done in triplicates. After incubation the number of motile planktons was counted and their percentage of mortality was recorded.

3 RESULTS

3.1 Characterization of coral-associated bacterial community

From the collected samples, a total of 429 microbial isolates were isolated and around 55 random bacterial isolates were chosen for the molecular characterization by 16S rRNA gene sequence amplification followed by subsequent phylogenetic identification. Through this, both the coral pathogenic and beneficial microbial communities were identified. The nature of the identified bacterial species was screened from literature and the roles of each identified bacterial species were recognized. The identified bacterial community is represented by Gammaproteobacteria (3 Genera, 6 species), Proteobacteria (2 Genera, 4 species) and Firmicutes (3 Genera, 7 species). The coral-beneficial bacterial genus was recommended to be *Pseudoalteromonas* (3 species), *Bacillus* (3 species), *Virgibacillus* (1 species) and *Cobetia* (2 species). Thus, a diverse group of bacterial species were identified from the host coral using molecular characterisation. The beneficial aspects of these isolates were further screened using their potential biomolecule production.

3.2 Characterization of potential coral beneficial microbes.

The potential coral-beneficial bacteria were initially screened by cross streak antagonistic method (Fig. 2 and 2a). The major seven coral-associated beneficial bacteria were identified among all the isolated bacterial strains for their significant inhibition zones against the tested coral and human pathogens (Fig. 2). They are *Pseudoalteromonas neustonica*, *Cobetia marina* 1, *Cobetia marina* 2, *Cobetia amphilecti* 4, *Cobetia amphilecti* 6, *Bacillus flexus* and *Virgibacillus halodenitrificans*. In the

cross streak method, *P. neustonica* exhibited inhibition activity against 6 pathogens apart from the other beneficial microbes. Whereas, *C. marina 2* and *V. halodenitrificans* revealed a maximum inhibition zone sizes of around 19 mm against *S. nematodiphila* (Fig. 2). Thus, through cross-streak analysis the potent coral beneficial microbes were screened and subjected to further extraction of the potent antimicrobial compound.

3.3 Intracellular and extracellular protein antagonism

The proteins extracted from the beneficial microbes were studied for their anti-pathogenic potential against various coral and human pathogens such as *S. nematodiphila*, *S. marcescens*, *P. alimentarius*, *P. pulmonis*, *B. paramycoids*, MRSA, *S. aureus*, *C. albicans*, *E. coli*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae*, *C. globosum*, and *A. sydowii*. Both intracellular and extracellular proteins of all the beneficial microbes had revealed inhibition against 6 out of the 15 tested pathogens (Fig. 3). *C. albicans* and *S. marcescens* were inhibited by *P. neustonica* and *V. halodenitrificans* respectively. Intracellular and extracellular proteins of *C. amphilecti* inhibited *V. vulnificus* apart from the intracellular proteins of *V. halodenitrificans*, *P. neustonica* and extracellular proteins of *C. marina*. Among all the extracellular proteins, *B. flexus* revealed inhibition against *S. aureus* alone. Although these beneficial microbial proteins presented inhibition against some pathogens, they failed to reveal activity against coral pathogens such as *A. sydowii*, *Vibrio* spp. and *S. marcescens*. Hence, they were subjected for bioactive metabolites extraction and were assayed against the pathogens of the study.

3.4 Intracellular and extracellular metabolite antagonism

The bioactive compounds of coral beneficial microorganisms inhibited all 15 studied coral and human pathogens significantly (Fig 4). In contrast to protein antagonism, the intracellular metabolite had no effect on all pathogens. The bioactive metabolites are then found to be produced extracellularly in the culture supernatant. These extracellular metabolites revealed antagonism at lower volumes/doses (25 µl) with significant inhibition zone sizes of up to 30 mm (Fig 5). Among all the seven beneficial microbes, *C. marina 1* and *B. flexus* revealed inhibition of only 2 (MRSA and *P. pulmonis*) and 3 (MRSA, *P. alimentarius* and *P. pulmonis*) tested pathogens respectively (Fig. 5). However, *P. neustonica* revealed inhibition of 11 pathogens, *V. halodenitrificans* inhibited 10 pathogens, *C. marina 2* inhibited 12 pathogens, *C. amphilecti 4* inhibited 12 pathogens and *C. amphilecti 6* inhibited 15 pathogens (Fig. 5). Thus, extracellular bioactive metabolites demonstrated a broader range of anti-bacterial effect than the proteins investigated. As a result, these compounds were tested for antagonism in bacterial broth cultures as well as against coral-associated protozoan ciliates and zooplanktons.

3.5 Metabolite antagonism in broth

The antagonism of coral associated beneficial bacterial metabolites was also tested in the broth culture against the pathogens, *V. harveyi* and *S. marcescens* (supplementary Fig. 1 and 2). The antibacterial activity of small molecules of *C. amphilecti* 6, *C. amphilecti* 4, *C. marina* 2, *P. neustonica* and *V. halodenitrificans* was significant against the coral infectious pathogens (supplementary Fig. 1 and 2). The metabolites dose greater than 25 µl displayed strong antagonism against coral pathogens. *S. marcescens* was strongly inhibited by *P. neustonica*, *C. marina* 2 and *C. amphilecti* 4. Comparatively, *S. marcescens* had displayed lesser inhibition than *V. harveyi*, which might be due to its higher cell density and virulence.

3.6 Metabolite antagonism on the protozoan ciliate

The antagonism of coral beneficial bacterial metabolites was tested against the coral disease-associated ciliates. Among the metabolite of seven coral beneficial bacteria examined, five beneficial bacterial metabolites of *C. amphilecti* 6, *C. amphilecti* 4, *C. marina* 2, *P. neustonica* and *V. halodenitrificans* revealed their strong activity against the ciliates, *Uronema marinum* and *Cohnilembus verminus* for complete mortality at a very lower dose of 10 µl, and a 20 µl dose of mortality with *Holosticha diademata* and *Euplotes vannus* (Fig. 6). However, *Cobetia marina* 1 (CM1) and *Bacillus flexus* (BFL) revealed less mortality rates of ciliates against all the four measured volumes. While the protozoan ciliates after treatment appeared inactive (Fig.7) and the cells were shrunken (Fig. 7) confirming its death.

3.7 Metabolite antagonism on the Zooplanktons

The secondary metabolites of coral beneficial bacteria had displayed significant antagonism against zooplanktons (Fig. 8). Among the various tested volumes of secondary metabolites, the metabolites of *P. neustonica*, *V. halodenitrificans* had revealed 60 µl dose to be lethal (supplementary Fig. 3). While the *C. amphilecti* 6 and *C. marina* 2 exhibited zooplankton mortality with 50 µl dose of secondary metabolites. However, *Cobetia marina* 1 (CM1) and *Bacillus flexus* (BFL) revealed less mortality rates of zooplanktons against all the four measured volumes when compared with that of the control. The abnormal bent abdomen and inactive conditions were observed with the secondary metabolites treated zooplanktons (supplementary Fig. 3). Thus, among the seven beneficial bacterial active metabolites tested, four revealed the antagonism activity against the zooplanktons that influences the disease severity of corals.

4 Discussion

Scleractinian corals are being tested for bacterial diversity in greater numbers [13, 46, 47-50] in order to better understand the coral susceptibility mechanism to heat and disease stress along with the coral microbial relationships [51]. At present, there is no viable cure to minimize the coral disease impacts and researchers are attentive in restoring the local population by gardening or propagation and selection of resistant communities of corals [52-54]. Thus, the advancement in treatments are to be employed *in situ* to enhance coral resilience and disease resistance [19]. Hence it is important to understand the coral microbial structure, interaction and functions together with their role in sustaining coral health. Microbes associated with corals have been demonstrated to protect host coral health through antimicrobial compound production against the infectious pathogens [55]. Although around 82 scleractinian coral species are reported in the coral reefs of the Gulf of Mannar and Palk Bay regions of Tamil Nadu coast, a biosphere reserve (UNESCO) of the Indian Ocean, which was not much explored for beneficial bacterial interaction for coral health [56]. Thus, the present study was attempted to examine the bioactive metabolites of coral beneficial bacteria against the various prokaryotic and eukaryotic pathogens (Bacterial, protozoan ciliates and zooplankton), which can enhance the coral health. Several studies have described coral as a bacterial source with antibacterial potential [57, 41], and also they are an undiscovered source of economically relevant microbial diversity [27, 44]. Bacterial symbionts of coral reefs secrete biological active secondary metabolites as a strategy to defend their host against various predators [58]. Besides, the biological active metabolites of coral associated bacteria are also demonstrated for application of human diseases such as anti-tumour, anti-microbial, anti-inflammatory, anti-protozoan, neuro suppressive and immunosuppressive properties together with gastrointestinal, respiratory and cardiac treatments [58]. Thus, in present investigation, 55 isolates with 18 species revealed a high bacterial diversity representing three major phyla such as Gammaproteobacteria, proteobacteria and firmicutes, supporting the other similar investigation with a high bacterial diversity in *Porites lutea* [46]. The presence of phylum Proteobacteria and Firmicutes was consistent with earlier studies [43, 59]. Seven bacterial isolates among the other isolated strains were found to produce bioactive antimicrobial metabolites that had the potential to inhibit the major reported coral pathogens such as *V. parahaemolyticus*, *V. vulnificus*, *Serratia marcescens*, *Aspergillus sydowii* and together with other clinical pathogens such as MRSA, *S. aureus* which are concurrent to the earlier reports [27, 60-62]. These seven bacterial isolates represented the genus *Pseudoalteromonas*, *Cobetia*, *Virgibacillus* and *Bacillus* which belong to well-known antibiotic producers. *Pseudoalteromonas* are commonly found in reef-building corals [42, 48, 63, 64]. They are notified to exhibit antimicrobial activity against both Gram-Negative and Gram-Positive bacteria [44,

65-67]. They exhibited antimicrobial activity against the known coral pathogen *Vibrio corallyticus* [19] and anti-pathogenic against Porites ulcerative white spot [68]. *Pseudoalteromonas* isolated from coral *O. patagonica* had inhibited coral pathogen *Vibrio shiloi* as well [43]. *Pseudoalteromonas* are considered to be versatile in the production of antimicrobial compounds including Secondary metabolite biosynthetic gene clusters (SM-BCGs) [69-70]. Furthermore, the *Pseudoalteromonas* associated with octocorals, *Eunicella verrucosa* and *Eunicella labiata* had revealed a SM-BGCs (RiPP-like) with antimicrobial activity against *Staphylococcus aureus*, *Salmonella enterica*, *Typhimurium* and *Vibrio parahaemolyticus* [71]. *Cobetia* species are known for bioindicator and marine probiotic in act of inducing resistance to corals during bleaching stress [19] was found beneficial in our study as well, by producing putative secondary metabolite production against the coral pathogens tested. The next abundant phylum was represented by Firmicutes with *Bacillus* spp. *Bacillus* species isolated from *Alcyonium digitatum*, *Bacillus* and *Pseudoalteromonas* associated with octocorals of Florida Keys, and *B. flexus* of *Porites* sp. had inhibited pathogens such as *Aurantimonas coralicida*, *V. shiloi*, *V. agarivorans*, *V. corallilyticus* and *S. marcescens* [72-74], which are agreeing with the *B. flexus* antibacterial activity of the present study. *B. flexus* also known to exhibit biosurfactants in anti-biofilm activity [73] and anti-pathogenic against Porites ulcerative white spot [68]. *B. firmus* associated to *Acropora* sp. has the ability to degrade chlorpyrifos [75]. *Virgibacillus salarius* and *Bacillus paramycoides* presented anti-pathogenic activity against the pathogens of urinary tract infection [76]. Therefore, beneficial bacteria that are associated with corals play an important role not only in preventing coral infections and supporting coral health, but they can also be very effective in battling infectious and non-infectious disorders in humans.

According to significant research, coral tissue damage is caused by marine ciliates that either directly or indirectly transmit disease [77-80]. Besides, they influence the disease severity on the immune-compromised coral tissues [81] and forage on the tissue lesions including the endosymbiont Symbiodiniaceae [82-84]. Thus, ciliates are generally believed to be opportunistic pathogens [85,86]. Numerous ciliates related to coral disease have been identified in the Caribbean and Indo-Pacific. The primary disease is Skeleton Eroding Band, which is caused by the folliculinid ciliate *Halofolliculina corallasia* [87]. The current study is the first of its type in demonstrating the bioactive metabolites of coral-beneficial bacterial activity against ciliates. The metabolites of four major bacteria such as *P. neustonica*, *C. marina*, *C. amphilecti* and *V. halodenitrificans* exhibited 100 % of mortality of all four (*Holosticha diademata*, *Cohnilembus verminus*, *Uronema marinum* and *Euplotes vannus*) coral-associated ciliates. Also, zooplanktons like Harpacticoid copepods are found to be more frequent associates of scleractinian corals [88]. Though there were no proven studies, they can also be suspected

to ingest the coral Symbiodiniaceae at stages of disease proliferation as they were collected from the dead coral tissues of the present study. Similar to ciliate inhibition the putative beneficial metabolites of coral-associated bacteria showed a mortality rate of more than 50 %. Altogether this study illustrated important coral-beneficial bacterial species metabolites producing a notable high antagonistic activity against the varied microbial pathogenic communities found dwelling on scleractinian corals. Thus, in reality, the antagonism activity of the metabolites against bacterial pathogens of the immune-compromised coral may help to gain its immunity and avoid the further proliferation of virulent bacterial community. This in consequence may reduce the reach of secondary pathogenic protozoan communities and promote coral disease resilience.

In conclusion, our results indicated that representatives of Gammaproteobacteria, Proteobacteria and Firmicutes associated with the coral *Porites lutea* had a broad antimicrobial spectrum. From a total of 420 isolates, 55 were characterized and based on the various antagonism screening the bioactive potential of four putative beneficial bacteria of corals were described. They displayed a remarkable antagonism against the coral associated bacterial, fungal and protozoan pathogens. Therefore, more in-depth research on the biopotentials of these metabolites at the compound level and *in vitro* studies may be useful in reducing the spread of coral disease.

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Conflict Of Interest Statement

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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FIGURE LEGENDS

FIGURE 1. Sample collection site of healthy, disease and algal dominated corals for isolation of associated microbes. Satellite image of the sample collection site at Gulf of Mannar, Tamil Nadu. Eukaryotic and prokaryotic microbes such as Bacteria, fungi, ciliates and zooplanktons samples were isolated from diseased, healthy and algal dominated coral *Porites lutea*.

FIGURE 2. Antibacterial activity screening of coral-associated beneficial bacteria (CABB) using cross streak method. Antagonistic activity of (A) *C. marina*, (B) *V. halodenitrificans* and (C) *B. flexus* against the pathogens (1) *S. aureus*, (2) *B. paramycoids*, (3) MRSA, (4) *V. vulnificus*, (5) *S. nematophila* and (6) *S. marcescens* and antagonistic activity of (D) *P. neustonica*, (E) *V. halodenitrificans*, (F) *B. flexus*, (G) *C. marina* and (H) *B. oceanisediminis* against pathogens of *Vibrio* species, (1) *Vibrio* sp. (14w), (2) *Vibrio* sp. (13w), (3) *Vibrio* sp. (11w), (4) *Vibrio* sp. (12w), (5) *Vibrio* sp. (3s), (7) *Vibrio* sp. (9w) and (6) *Candida albicans*.

FIGURE 2a. Zone of inhibition measurements of antagonistic activity of the coral-associated beneficial bacteria against bacterial pathogens using cross streak method. Isolated potential beneficial bacteria such as PN (*P. neustonica*), CM1 (*C. marina* 1), CM2 (*C. marina* 2), VG (*V. halodenitrificans*), CA4 (*C. amphilecti* 4), CA6 (*C. amphilecti* 6), BFL (*B. flexus*), BO (*B. oceanisediminis*), PC (*Pseudoalteromonas carrageenovora*), PE (*P. espijana*) inhibited the coral pathogens in a cross streak method. The zone of inhibition attained is represented in millimeters.

FIGURE 3. Antagonism activity of the peptides of coral-associated beneficial bacteria. The activity of intracellular (I) and extracellular (E) peptides of PN (*P. neustonica*), CM1 (*C. marina* 1), CM2 (*C. marina* 2), VG (*V. halodenitrificans*), CA4 (*C. amphilecti* 4), CA6 (*C. amphilecti* 6), BFL (*B. flexus*) was screened against pathogens. The zone of inhibition attained by well diffusion assay are represented in millimeters.

FIGURE 4. Antagonistic activity of the secondary metabolites of beneficial bacteria using well diffusion method. The activity of secondary metabolites of beneficial bacteria such as PN (*P. neustonica*), CM1 (*C. marina* 1), CM2 (*C. marina* 2), VG (*V. halodenitrificans*), CA4 (*C. amphilecti* 4), CA6 (*C. amphilecti* 6), BFL (*B. flexus*) against the pathogens A- *S. marcescens*, B- *V. cholerae*, C- *A. sydowii*.

FIGURE 5. Zone of inhibition measurements of antimicrobial activity of secondary metabolites of the coral-associated beneficial bacteria against pathogens. The antagonism activity of extracellular metabolites extracted from beneficial bacteria such as PN (*P. neustonica*), CM1 (*C. marina* 1),

CM2 (*C. marina* 2), VG (*V. halodenitrificans*), CA4 (*C. amphilecti* 4), CA6 (*C. amphilecti* 6), BFL (*B. flexus*) was screened against the coral and human pathogens. The zone of inhibition obtained by well diffusion assay are represented in millimeters.

FIGURE 6. Antagonism activity of the metabolites of coral beneficial bacteria against eukaryotic ciliates. The activity of metabolites extracted from beneficial bacteria such as PN (*P. neustonica*), CM1 (*C. marina* 1), CM2 (*C. marina* 2), VG (*V. halodenitrificans*), CA4 (*C. amphilecti* 4), CA6 (*C. amphilecti* 6), BFL (*B. flexus*) against the ciliates, *Uronema marinum*, *Euplotes vannus*, *Cohnilembus verminus*, and *Holosticha diademata*.

FIGURE 7. Protozoan ciliates treatment with metabolites of coral beneficial bacteria. Healthy adult ciliates such as 1. *Cohnilembus verminus*, 2. *Euplotes vannus*, 3. *Uronema marinum*. 1A- *C. verminus* control, 1B – *C. verminus* treated were observed with cells shrink to death., 2C- *E. vannus* control, 2D – *E. vannus* treated were observed with cells shrink to death, and 3E – *U. marinum* control and 3F – *U. marinum* treated were observed with cells shrink to death (arrow marked).

FIGURE 8. Antagonism activity of the metabolites of coral beneficial bacteria against the Zooplanktons isolated from the coral disease lesions. The activity of beneficial bacteria such as *P. neustonica* (PN), *V. halodenitrificans*, (VG), *C. amphilecti* 6 (CA6), *C. marina* 2 (CM2), *C. marina* 1 (CM1), *Bacillus flexus* (BFL), control (cntrl).

SUPPLEMENTARY FIGURE

FIGURE 1. Antimicrobial activity of metabolites of coral beneficial bacteria in broth condition. The activity of beneficial bacteria such as PN (*P. neustonica*), CM1 (*C. marina* 1), CM2 (*C. marina* 2), VG (*V. halodenitrificans*), CA4 (*C. amphilecti* 4), CA6 (*C. amphilecti* 6), BFL (*B. flexus*) against the broth culture of *S. marcescens*.

FIGURE 2. Antimicrobial activity of metabolites of coral beneficial bacteria in broth condition. The activity of beneficial bacteria such as PN (*P. neustonica*), CM1 (*C. marina* 1), CM2 (*C. marina* 2), VG (*V. halodenitrificans*), CA4 (*C. amphilecti* 4), CA6 (*C. amphilecti* 6), BFL (*B. flexus*) against the broth culture of *V. harveyi*.

FIGURE 3. Antagonism of metabolites of coral beneficial bacteria against coral-associated zooplanktons. Zooplanktons were observed with ingested coral-associated symbiotic algae and the dead coral tissues. After treatment with metabolites, the zooplanktons were observed for abnormal bent abdomen and inactive conditions.

FIGURE 1

Satellite Image



Diseased *Porites lutea*



Healthy *Porites lutea*



Algal dominated *Porites lutea*

FIGURE 2

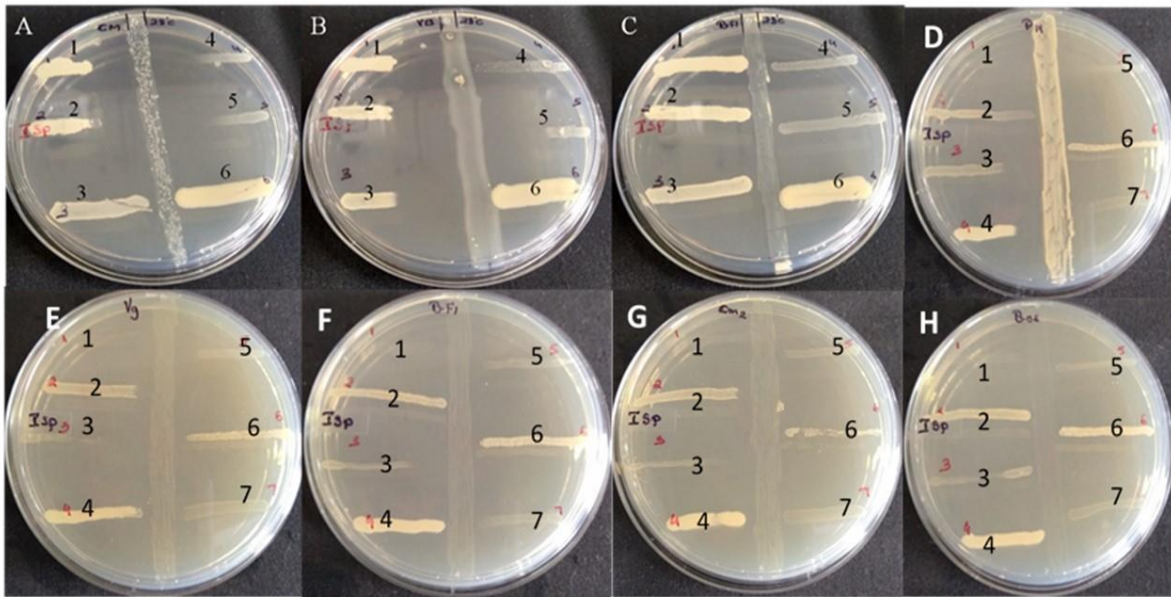


FIGURE 2a

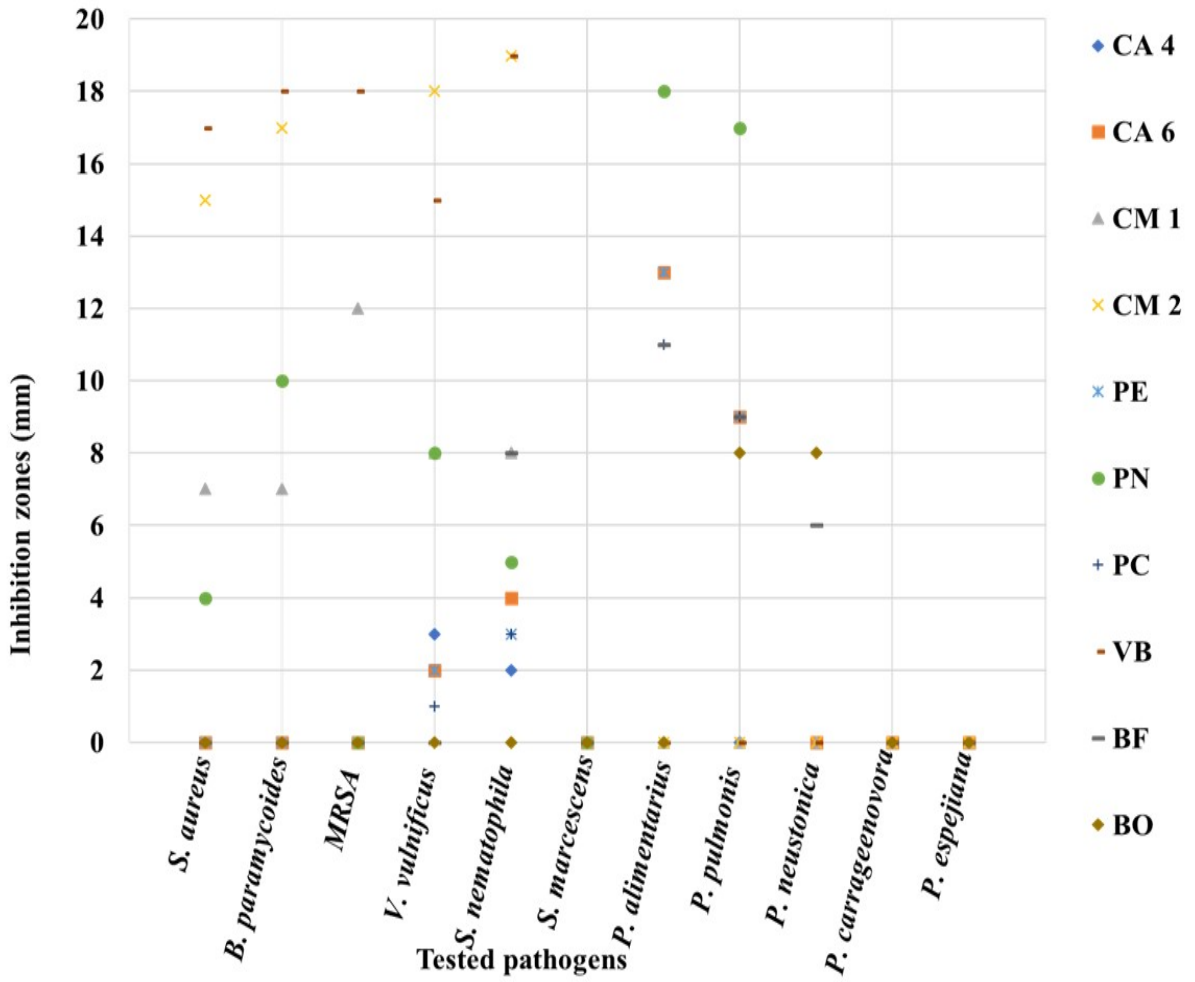


FIGURE 3

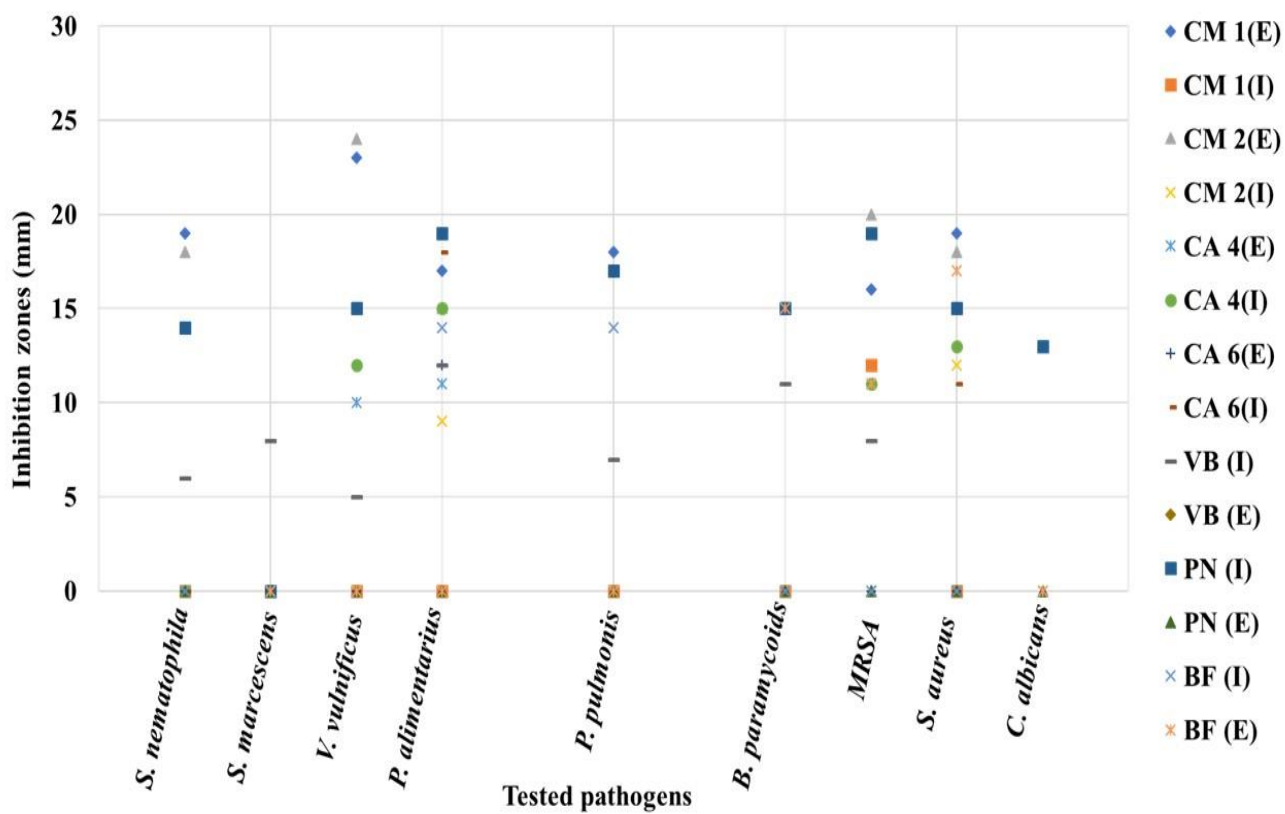


FIGURE 4

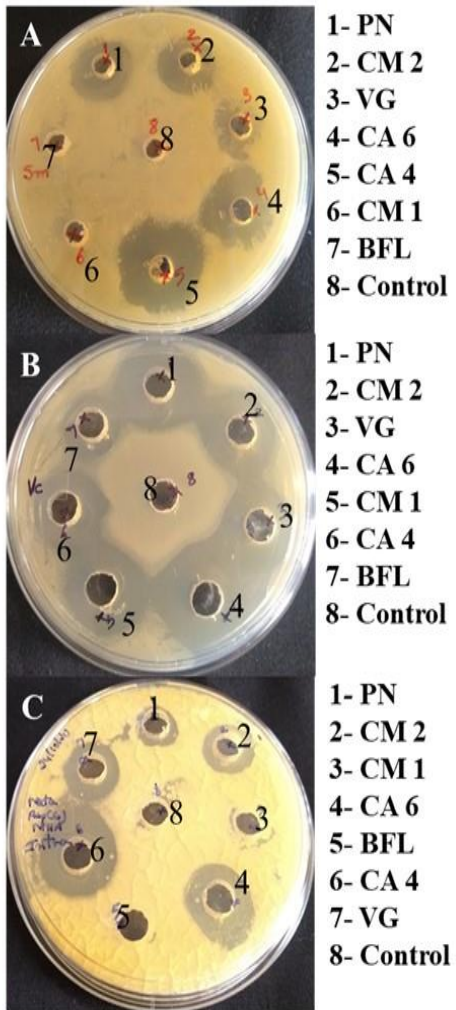


FIGURE 5

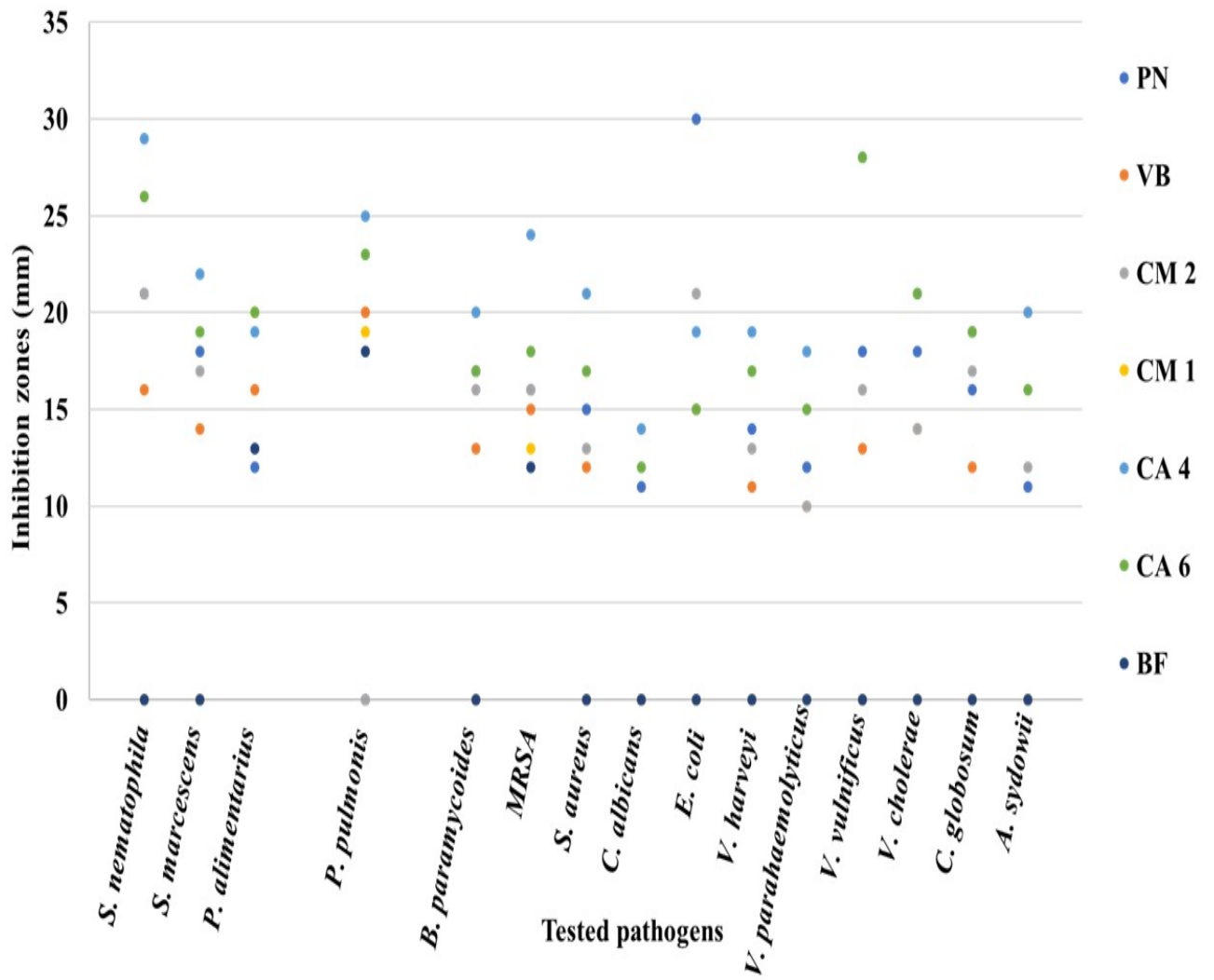


FIGURE 6

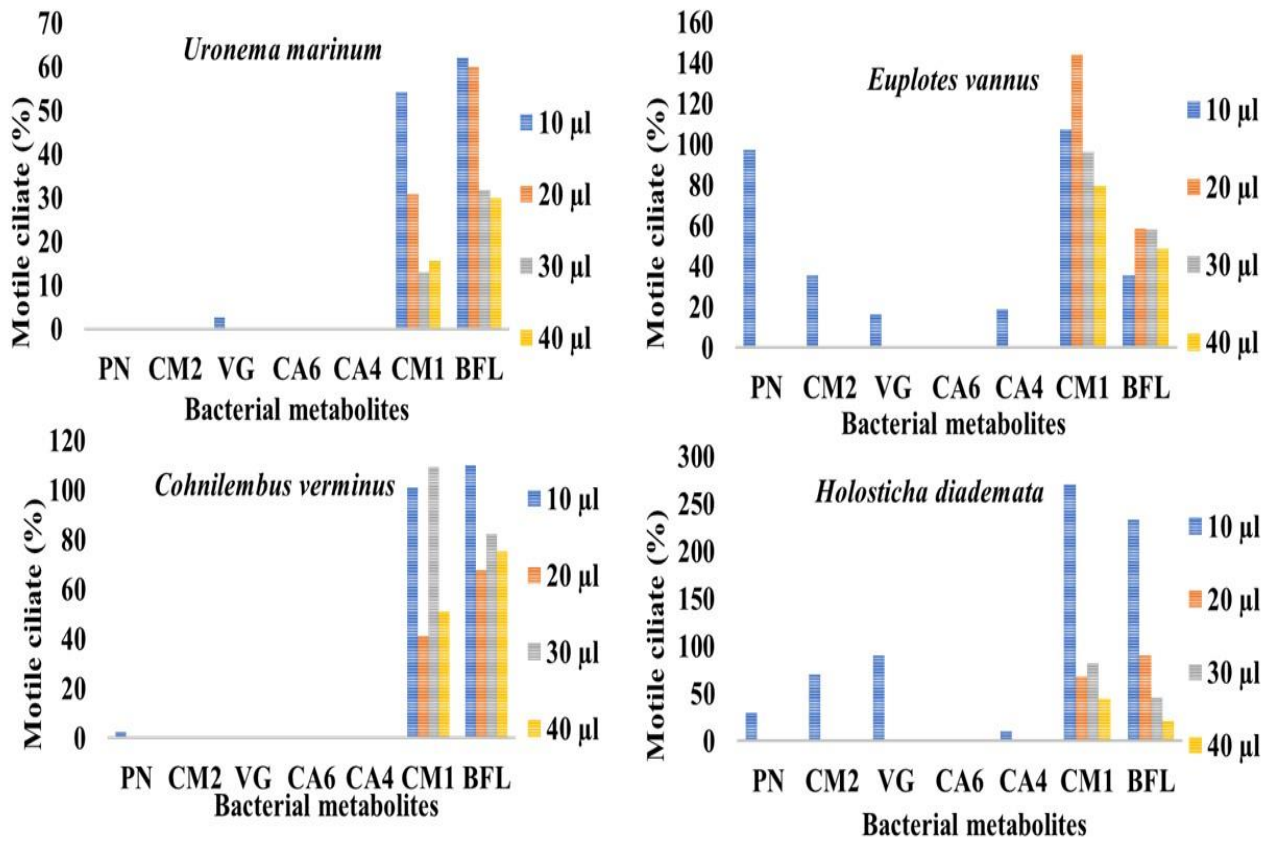


FIGURE 7

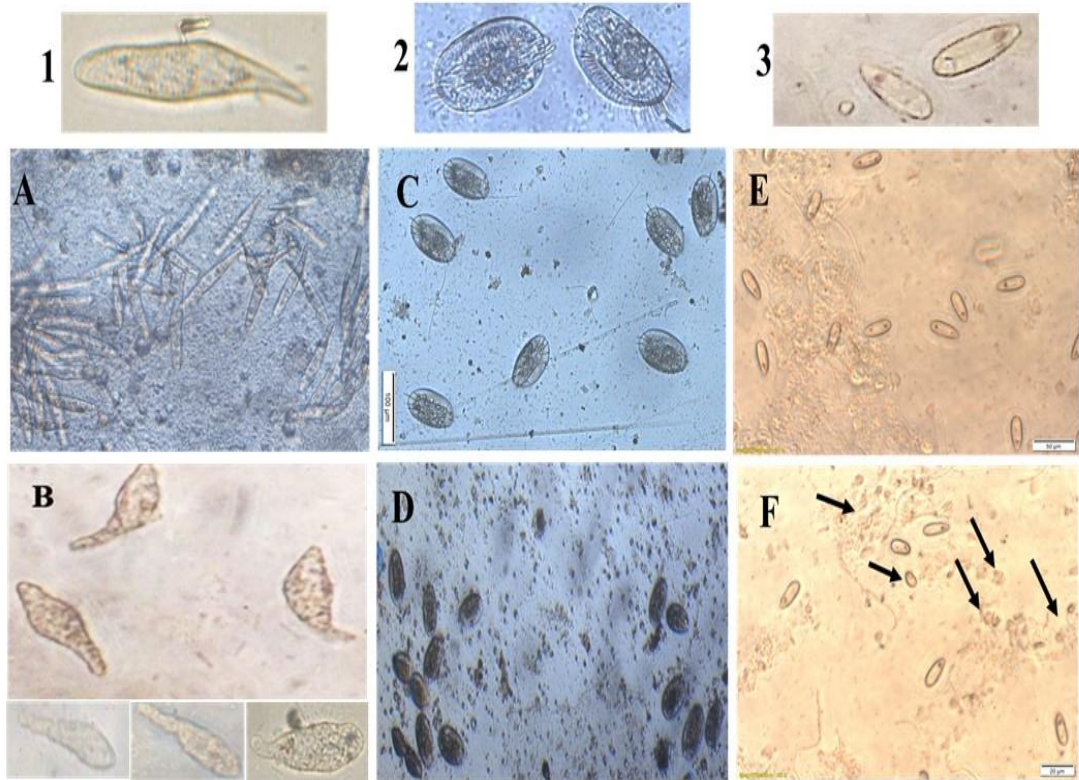
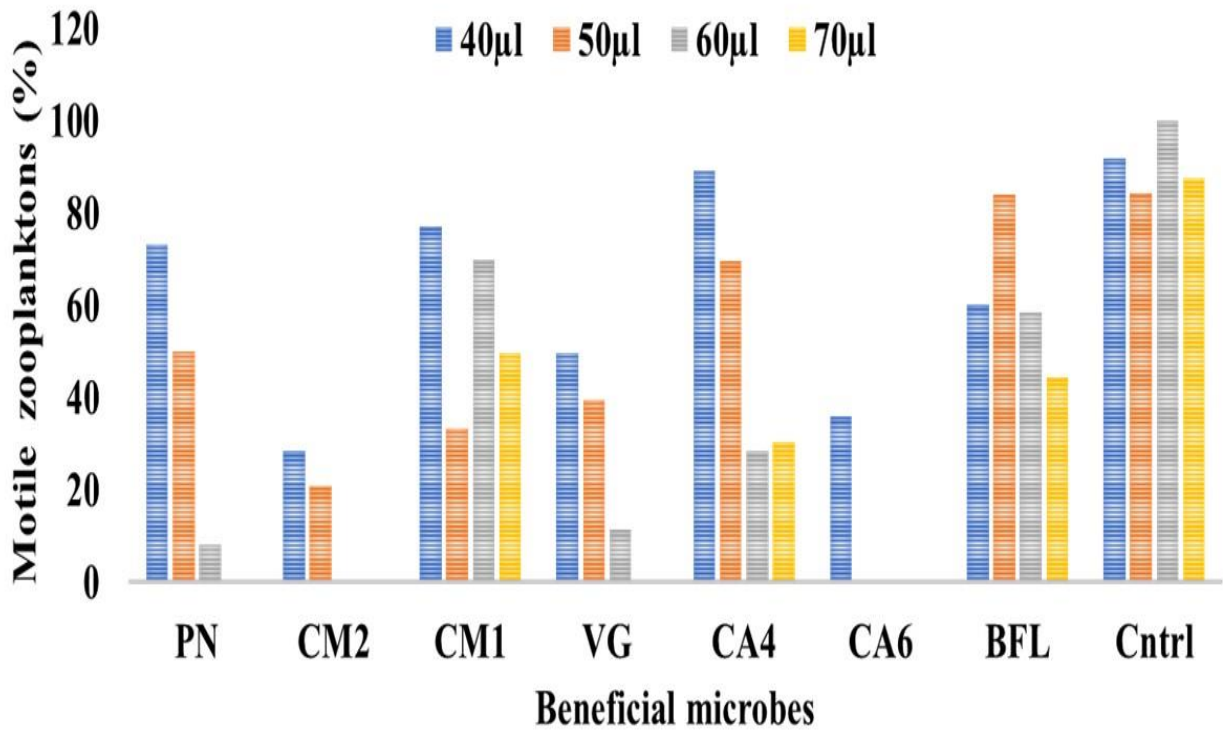
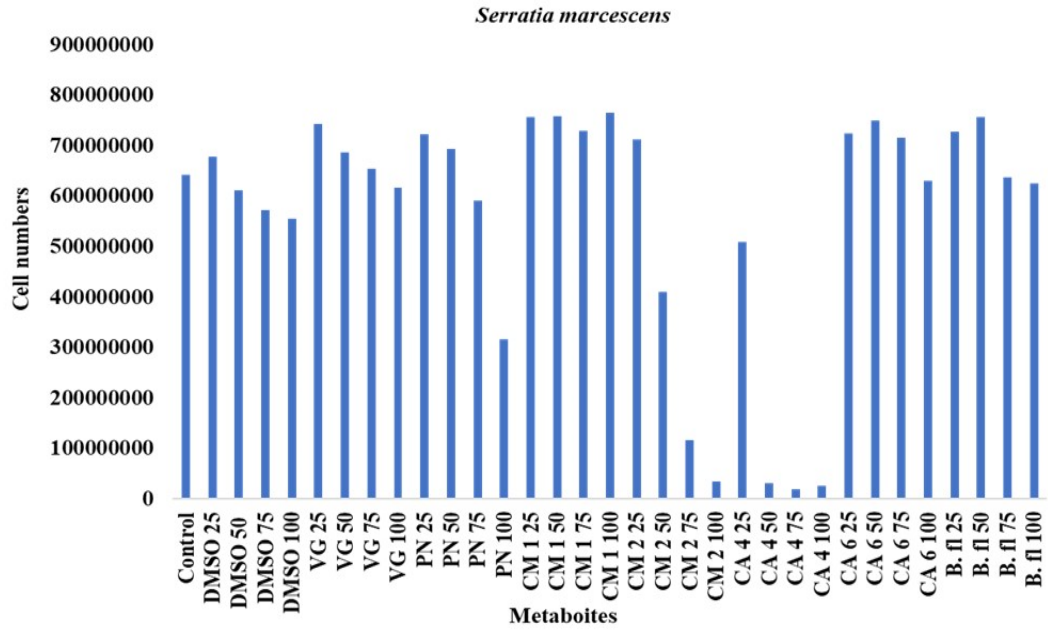


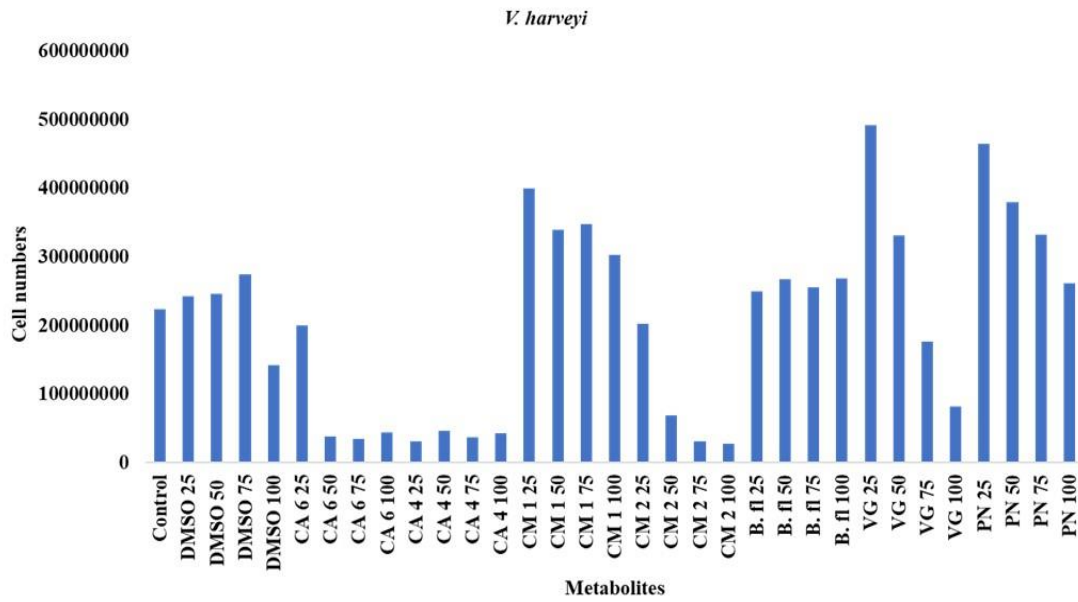
FIGURE 8



Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3

