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1 **Phylogenetic diversity and activity screening of cultivable actinobacteria isolated**
2 **from marine sponges and associated environments from the western coast of India**
3

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22 *Keywords:* Bacterial predation; Antibiotic production; Secondary metabolites; Enzyme
23 inhibition; Molecular phylogeny.
24

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25 **Abstract**

26 Phylogenetic diversity of cultivable actinobacteria isolated from sponges and associated
27 environments of intertidal zones, along the northern parts of west coast of India, were studied
28 using 16S rRNA gene sequences. A subset of actinobacteria were screened for three activities,
29 namely predatory behavior, antibacterial activity and enzyme inhibition. We recovered 207
30 isolates of actinobacteria belonging to 16 families and 25 genera, which could be attributed to
31 55 putative species using Poisson Tree Processes and 60 putative species based on Bayesian
32 Poisson Tree Processes. Although the trends in the discovery of actinobacterial genera isolated
33 from sponges was consistent with previous studies from different study areas, we provide first
34 report of six actinobacterial species from sponges. We observed widespread non-obligate
35 epibiotic predatory behavior in eight actinobacterial genera and we provide first report of
36 predatory activity in *Brevibacterium*, *Glutamicibacter*, *Micromonospora*, *Nocardiopsis*,
37 *Rhodococcus* and *Rothia*. Sponge associated actinobacteria showed significantly more
38 predatory behavior than environmental isolates. While antibacterial activity by actinobacterial
39 isolates mainly affected Gram-positive target bacteria with little to no effect on Gram-negative
40 bacteria, predation targeted both Gram-positive and Gram-negative prey with equal propensity.
41 Actinobacterial isolates from both sponge and associated environment produced inhibitors of
42 serine proteases and angiotensin converting enzyme. Predatory behavior was strongly
43 associated with inhibition of trypsin and chymotrypsin. Our study suggests that sponge and
44 associated environment of western coast of India are rich in actinobacterial diversity with
45 widespread predatory activity, antibacterial activity and production of enzyme inhibitors.
46 Understanding diversity and associations among various actinobacterial activities, with each
47 other and the source of isolation, can provide new insights in marine microbial ecology and
48 provide opportunities to isolate novel therapeutic agents.

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49 **INTRODUCTION**

50 The marine ecosystem is not only diverse with respect to microorganisms found in it but also
51 the natural products being synthesized by these microorganisms (Ward and Bora, 2006; Taylor
52 et al., 2007; Lam, 2006). Actinobacteria are among the taxa rich in secondary metabolites
53 (Barka et al., 2016) and are widely distributed in diverse habitats including soil, marine and
54 freshwaters and sediments (Ward and Bora, 2006; Taylor et al., 2007; Tan et al., 2015; Brasel
55 et al., 2019; Mincer et al., 2002; Kokare et al., 2004). They are also not uncommon in extreme
56 environments (Jose and Jebakumar, 2014; Pathom-Aree et al., 2006; Mohammadipanah and
57 Wink, 2016; Shivlata and Tulasi, 2015; Riquelme et al., 2015; Yang et al., 2015) and are also
58 found as endobiotic symbionts of higher organisms (Taylor et al., 2007; Li et al., 2015;
59 Mahmoud and Kalendar, 2016; Trujillo et al., 2015). They belong to the phylum Actinobacteria
60 and represent one of the major phyla within the bacterial domain (Goodfellow, 2015). They are
61 aerobic, spore forming, Gram-positive bacteria, which often produce diffusible pigments, and
62 occur as cocci or rods, branched filaments, aerial or substrate mycelium (Goodfellow, 2015).
63 The marine ecosystems are believed to have a wide range of unexplored diversity of
64 actinobacteria (Montalvo et al., 2005) and their metabolites (Taylor et al., 2007; Lam, 2006;
65 Manivasagan et al., 2005) with diverse biological activities like anticancer (Olano et al., 2009),
66 anti-inflammatory (Trischman et al., 1994), antibiotic (Pimentel-Elardo et al., 2010; Cheng et
67 al., 2015; Gandhimathi et al., 2008), cytotoxic (Abdelfattah et al., 2016) and enzyme inhibitory
68 (Manivasagan et al., 2015; Imada, 2005) activity. Watve et al. (2001) estimated that the genus
69 *Streptomyces* alone is capable of producing up to 10^5 different metabolites, majority of which
70 remain unexplored. Of 23,000 medicinally important metabolites produced by marine
71 microorganisms 70% are contributed by actinobacteria (Mahapatra et al., in press). Till date,
72 eight genera of actinobacteria have been reported to produce secondary metabolites and 267
73 products have been reported from 96 marine actinobacteria (Subramani and Sipkema, 2019)[31].

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74 Ecologically it is difficult to understand the production of extracellular metabolites or
75 enzymes by aquatic bacteria, since any molecule secreted outside the cell can be quickly
76 washed off. Extracellular products could be useful to the producer only in viscous or partially
77 enclosed environments. In the marine environment, sponges are likely to provide such closed
78 environment for bacteria. Sponges are filter feeders and collect small nutrient particles
79 including bacteria. This makes the environment locally nutrient rich in an otherwise
80 oligotrophic surroundings. Bacteria, especially actinobacteria, isolated from these sponges may
81 live in a symbiotic relationship that helps the host in defense against predation, sponge skeleton
82 stabilization, translocation of metabolites and help in nutritional process (Taylor et al., 2007;
83 Li et al., 2015; Montalvo et al., 2005; Pimentel-Elardo et al., 2010; Cheng et al., 2015;
84 Gandhimathi et al., 2008; Lee et al., 2009; Thomas et al., 2010). In addition, since sponges are
85 sessile and lack other anti-predator defenses, secondary metabolites of bacteria can provide
86 them with chemical defense (Lee et al., 2001). Therefore, we expect more secondary metabolite
87 related activities from sponge-associated actinobacteria.

88 Sponge-associated actinobacteria are likely to have another ecological role. Among
89 actinobacteria at least three genera, namely *Agromyces*, *Streptomyces* and *Streptoverticillium*,
90 are shown to be predators that kill and feed on other live bacterial cells (Casida, 1980; 1983;
91 Kumbhar et al., 2014). Kumbhar and Watve (2013) argued that antibiotic activity might have
92 evolved primarily as a weapon in predation. However, the expression of secondary metabolites
93 during predation may be independent of antibiotic expression in pure culture; the latter is likely
94 to have evolved for mutualism with higher animal or plant hosts (Harir et al., 2018; Van der
95 Meij et al., 2017). Further, for a niche of predation in association with sponge, the predatory
96 species needs to protect itself from the digestive enzymes of the sponge as well as its own
97 enzymes used for predation. Therefore, predatory actinobacteria are also expected to have
98 efficient inhibitors of lytic enzymes.

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99 In this study, we prepared an inventory of cultivable actinobacteria from sponges and
100 associated environments of intertidal zones along the northern parts of west coast of India and
101 studied their molecular diversity based on 16S rRNA gene sequences. We screened a subset of
102 randomly selected cultures for predatory activity, antibiotic production and enzyme inhibition
103 and tested their associations with each other and with the isolation source to test the hypotheses
104 mentioned earlier.
105
106 **MATERIALS AND METHODS**
107 **Sample collection**
108 Small tissue samples (less than one gram) of marine sponges were collected at the time of low
109 tide along Maharashtra and Goa coast (18–15ºN and 73–74ºE) of India during April 2014 to
110 October 2018 without damaging the sponge or its associated environment. Specimens were
111 rinsed and flushed with sterile media to remove debris and loosely attached microbes. Each
112 sponge sample was collected in labeled polystyrene tubes with lids containing sterile Poor
113 Ravan Saline (Watve et al., 2000) and ZoBell Marine broth (ZoBell, 1941). Sediment, water
114 and air samples were collected from the same environment as that of the sponge and were
115 collectively considered as environmental samples. The samples were brought to laboratory
116 maintaining cold chain and were immediately processed for microbial culturing.
117
118 **Isolation and maintenance of cultivable actinobacteria**
119 Each sample was subjected to pre-heat treatment at 60°C for 15 minutes to eliminate non-
120 sporulating bacteria. Sponge tissue (0.1 cm^3) was homogenized in sterile medium and vortexed
121 for 5 minutes. Tubes were left undisturbed for two minutes. From the resulting supernatant
122 serial 10 fold dilutions upto 10^{-5} were made and 0.1 ml sample was spread into triplicates on
123 petri plates containing sterile medium. We used two media the Zobell Marine Agar (ZMA) and

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124 Poor Ravan Saline Agar (PRSA) with and without antibiotic chloramphenicol (25 µg/ml).
125 Plates were incubated at 30°C for 7 days in the case of ZMA and 21 days for PRSA. Plates
126 were observed regularly for the growth of actinobacteria. Bacterial colonies that showed
127 resemblance to actinobacteria under light microscope were purified several times on the
128 respective media. In all 207 actinobacterial isolates were selected and were re-streaked for
129 making pure cultures. Colonies were labeled as per Maharashtra Gene Bank (MGB) project
130 code and preserved on ZMA slants at 4°C for further use. Similarly, glycerol (18%) stocks
131 were prepared and maintained at -20°C for long term storage. Actinobacterial cultures are
132 deposited in the Microbial Culture Collection (MCC) of National Centre for Microbial
133 Resource, National Center for Cell Sciences, Pune, India (accession numbers are provided in
134 the Supplementary Table S1).

135

136 **Genetic identification, phylogeny and species delimitation**

137 Actinobacterial isolates were outsourced for near complete 16S rRNA gene sequencing. Gene
138 sequences used for the study are deposited in the GenBank database under the accession
139 numbers MN339687–MN339897. Sequences were checked in BLAST (Altschul et al., 1990)
140 to find the closest sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov).
141 Four species of Firmicutes, namely *Bacillus paralicheniformis* (MCC 6306), *B. thuringiensis*
142 (MCC 7835), *B. subtilis* (MCC 6386) and *B. halotolerans* (MCC 8381), were used as outgroups
143 (GenBank accession numbers MN339894–MN339897 respectively).

144 Gene sequences were aligned using MUSCLE (Edgar, 2004) implemented in MEGA 7
145 (Kumar et al., 2016). Final aligned matrix had 1595 sites. Best nucleotide substitution model
146 was determined using ModelFinder (Kalyaanamoorthy et al., 2017) based on Bayesian
147 information criterion (Schwarz, 1978; Nei and Kumar, 2000). Maximum likelihood analysis

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148 was performed in IQ Tree (Nguyen et al., 2015) with ultrafast bootstrap support (Hoang et al.,
149 2018) for 1000 iterations. Phylogenetic tree was edited in FigTree v1.4.2 (Rambaut, 2009).
150 To understand putative number of actinobacterial species we performed species
151 delimitation based on Poisson Tree Processes (PTP) and Bayesian Poisson Tree Processes
152 (bPTP) methods (Zhang et al., 2013). Maximum likelihood tree was used to delimit species by
153 setting the parameter values as follows: MCMC generations = 100,000, Thinning = 100, Burn-
154 in = 0.1 and seed = 123.
155 We have identified all isolates up to genus level, while operational taxonomic units, in
156 terms of putative species, are provided based on PTP and bPTP methods (see Supplementary
157 Table S1). Only in the text, some isolates are assigned to known species based on BLAST
158 search and sequence identity more than 99%.
159

160 **Screening for activities**

161 Out of 207 actinobacterial isolates, 49 isolates were randomly selected for screening of three
162 activities, namely predation, antibiotic production and production of enzyme inhibition.
163

164 **Target bacteria used for predation and antibiotic screening**

165 Test bacteria, used for checking actinobacterial predation and antibiotic production, were
166 obtained from MCC and National Collection of Industrial Microorganisms (NCIM), National
167 Chemistry Laboratory, Pune, India. Fourteen bacteria, namely *Acetobacter pasterianus* (NCIM
168 2317), *Alcaligenes fecalis* (NCIM 2262), *Bacillus subtilis* (NCIM 2063), *Enterobacter fecalis*,
169 *Escherichia coli* (NCIM 2184), *Klebsiella pneumonae* (NCIM 2957), *Micrococcus luteus*
170 (NCIM 2673), *Mycobacterium smegmatis* (NCIM 5138), *Proteus vulgaris* (NCIM 2172),
171 *Pseudominas aeruginosa* (NCIM 5029), *Salinicoccus roseus* (MCC 7574), *Salmonella*

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172 *enterica* (NCIM 2501), *Serretia marcescens* (NCIM 2919) and *Staphylococcus aureus* (NCIM
173 2121), were used as target species for screening.
174
175 **Screening for actinobacterial predatory growth**
176 Growth of predator with the zone of clearance on prey cells was considered as predation as
177 defined earlier (Kumbhar et al., 2014). The method for the preparation of prey cells was
178 modified from Kumbhar et al. (2014). Pure cultures of the prey species were inoculated on
179 nutrient agar plates to check the purity and were later re-inoculated in nutrient broth. Inoculated
180 flasks were incubated at 37°C for 24 h. Broth was centrifuged at 7000 rpm for 10 minutes to
181 concentrate cells using Eppendorf centrifuge 5810R. Cells were washed thrice with sterile
182 distilled water to remove traces of nutrient broth. Pellet was suspended in saline to obtain a
183 thick suspension of optical density of 1.0 at 600 nm. Lawn of prey cells was spread on water
184 agarose plate and plates were incubated at 37°C for 40 minutes. Actinobacterial culture was
185 spot inoculated on pre incubated plates. These plates were incubated at room temperature for
186 48–72 h at 30°C. Plates with plaque were examined visually and by using 4x and 45x
187 magnification under light microscope. Prey and predator control plates were used for
188 comparison. Each experiment consisted of triplicate sets of plates, as well as one predator
189 control for testing growth of actinobacterial predator without prey. In addition, there was a prey
190 control to demonstrate viable and independent growth of prey without predator. In either
191 controls there was no zone of clearance indicating there was no predation in the presence of
192 predator or prey alone.
193
194 **Screening for antibacterial activity using conventional cross streak method**
195 Selected actinobacterial cultures were screened for antibacterial activity by cross streak method
196 (Velho-Pereira and Kamat, 2011; Valli et al., 2012). Test organism was streaked as a straight

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197 line along the diagonal of the petri dish with sterile ZMA medium. The isolated pure colony of
198 actinobacteria was inoculated as a single streak perpendicular to the central streak. Streaking
199 was done from the edge of the plate to the test organism growth line. Plates were incubated at
200 37°C for 18 h. The microbial inhibition was observed by determining zone of clearance around
201 the sensitive organisms. Control plates of the same medium with the streak of test bacteria and
202 without the streak of actinobacteria growth was used to observe the normal growth of the test
203 bacteria.
204
205 **Screening for enzyme inhibitors**
206 Actinobacterial cultures were screened for their ability to inhibit the activity of serine proteases
207 and angiotensin converting enzyme (ACE). Three different serine proteases i.e., Subtilisin,
208 Trypsin and α-Chymotrypsin were used for screening of inhibitory activity. Protease inhibitor
209 activity was studied using unprocessed X-ray films and spot-test method (Cheung, 1991) with
210 modifications. As described by Tripathi et al. (Tripathi et al., 2011), dilutions of pure enzyme
211 were first spotted on gelatine coated films. Lowest dilution showing complete clearance
212 (indicating complete digestion of gelatine) was chosen for further studies. Pure enzyme (100
213 µg/ml) was incubated with equal quantity of cell free supernatant of actinobacterial isolates for
214 10 minutes and transferred to untreated X-ray-Fuji Medical X-ray, HRU grade-films. The
215 mixtures were allowed to react for 15 minutes at room temperature and results were recorded
216 after washing the x-ray films under running water. Unprocessed X-ray films contain a layer of
217 gelatine on their surface, which acts as a substrate for various proteolytic enzymes. Degradation
218 of gelatine gives a clear zone at the site of activity. Thus, upon action of the proteases, clear
219 zones were seen on unprocessed x-ray films, at the site of inoculation, whereas, if the gelatine
220 layer remains intact, no clearance is observed. No clearance on the films indicated presence of
221 protease inhibitors.

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222 ACE acts on a specific substrate N-Hyppuryl-His-Leu (HHL) to liberate hippuric acid
223 and His-Leu. Liberated hippuric acid was detected spectrophotometrically. Upon reaction of
224 the enzyme with ACE inhibitors, the enzyme becomes inactive and this is measured in terms
225 of lower levels of hippuric acid released. Protocol suggested by Cushman and Cheung (1971)
226 was used with certain modifications and hippuric acid liberated was checked using method
227 suggested by Ng et al. (2008). Equal amount of ACE and cell free supernatants (10 µl each)
228 were allowed to react at 37°C. After 10 minutes 20 µl of HHL was added to the reaction mixture
229 and reaction was continued for 30 mins at 37°C. The reaction was stopped by addition of 40 µl
230 of 1 N HCl. Blank was prepared by addition of HCl before addition of the substrate. Positive
231 enzyme control was prepared by incubating enzyme with un-inoculated broth. Liberated
232 hippuric acid was extracted in 90 µl ethyl acetate by vigorous shaking. Ethyl acetate layer was
233 collected in a fresh vial and allowed to dry in water bath of 50°C. The liberated hippuric acid
234 was diluted in 150 µl distilled water and absorbance was checked at 228 nm. Zero was adjusted
235 using distilled water. Test vials with more than 15% inhibition of ACE were considered as
236 positive for ACE inhibitor.

237

238 **RESULTS**

239 **Actinobacterial phylogenetic diversity in sponge and associated environment**

240 Actinobacteria from sponges and associated environments showed a rich phylogenetic
241 diversity (Figure 1). We obtained 207 actinobacterial isolates, from sponge and associated
242 environments, belonging to 16 families and 25 genera (Supplementary Table S1). Species
243 delimitation based on Poisson Tree Processes (PTP) suggested that these isolates belong to 55
244 putative species, while Bayesian Poisson Tree Processes (bPTP) suggested 60 putative species.
245 The two species delimitation methods, PTP and bPTP, differed in the groups of species under
246 genera *Brevibacterium*, *Kocuria*, *Microbacterium* and *Streptomyces* (Supplementary Table

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247 S1). Air was generally devoid of actinobacteria and we recovered only three isolates from air,
248 belonging to genera *Brachybacterium*, *Brevibacterium* and *Rhodococcus*, as compared to 36
249 isolates from water, 90 isolates from sediment and 78 isolates from sponge.

250 From sponges, 15 genera under 11 families belonging to 30 putative species based on
251 PTP and 33 putative species based on bPTP were recorded (Figure 1, Table 1). From the
252 sponge-associated environment, 21 genera under 14 families were recorded belonging to 40
253 putative species as per PTP and 42 putative species as per bPTP. A total of 11 genera under 8
254 families and 15 putative species that were common to both sponge and associated environment.
255 Four genera, namely *Gordonia*, *Mycolicibacterium*, *Pseudonocardia* and *Rothia* were isolated
256 only from sponges (Table 1), which could be identified to species *Gordonia terrae* (MCC
257 6452), *Mycolicibacterium poriferae* (MCC 6242), *Pseudonocardia kongjuensis* (MCC 7930)
258 and *Rothia terrae* (MCC 7823) respectively. Although 11 genera, namely *Agrococcus*,
259 *Arthrobacter*, *Brachybacterium*, *Brevibacterium*, *Kocuria*, *Microbacterium*, *Micrococcus*,
260 *Micromonospora*, *Nocardiopsis*, *Rhodococcus* and *Streptomyces*, were isolated from both
261 sponges and associated habitats, most of these genera had some putative species that were
262 either exclusive to sponges or associated environments. In particular, 7 species,
263 *Brachybacterium muris* (MCC 7614), *Brevibacterium casei* (MCC 6140, MCC 6152, MCC
264 6176), *Kocuria rhizophila* (MCC 8384), *Nocardiopsis salina* (MCC 7931), *Rhodococcus zopfii*
265 (MCC 7934), *Streptomyces smyrnaeus* (MCC 7924) and *Streptomyces viridobrunneus* (MCC
266 7990), were recorded only from sponges.

267 With respect to both, the number of isolates and number of putative species,
268 *Streptomyces* was the most dominant genus, which was found in both sponges and associated
269 environments. *Nocardiopsis* was the second most common genus with two dominant species
270 *Nocardiopsis alba* (MCC 8385) followed by *N. dassonvillei* (MCC 7845). Other genera, which
271 were present in both sponge and environment include *Agrococcus*, *Arthrobacter*,

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272 *Brevibacterium*, *Kocuria*, *Microbacterium* and *Micrococcus*. Among the genera and species
273 that were recorded only from the environment, we provide first record of species such as
274 *Aeromicrobium massiliense* (MCC 6739) and *Glutamicibacter mysorens* (MCC 7825) from
275 marine waters.
276
277 **Non-obligate epibiotic predatory activity.** Out of the total 49 actinobacterial isolates
278 screened for non-obligate epibiotic predatory activity, 26 isolates showed predation on at least
279 one of the 14 target organisms (Supplementary Table S2). Of the 26 isolates with predatory
280 behavior, 17 preyed on Gram-negative prey, 21 preyed on Gram-positive prey, while 12 preyed
281 on both Gram- negative and Gram-positive prey. There was no significant difference (Mann-
282 Whitney U = 15, P = 0.2601) in the frequency of actinobacterial predators on Gram-negative
283 and Gram-positive prey (Table 2). Most actinobacterial predators (n = 14) preyed on a single
284 prey species while only a few predators preyed on multiple prey species with just a single
285 predator of the genus *Streptomyces* that preyed on 8 prey species. There was a significant
286 association between the source of isolation (sponge or associated environment) and predatory
287 behavior (χ^2 = 6.200, P = 0.0128), where the isolates from sponge showed proportionately
288 more predatory behavior (Figure 2).
289 All eight isolates of *Streptomyces* used for screening showed predatory behavior and
290 preyed on both Gram-negative and Gram-positive prey (Supplementary Table S2). Out of 25
291 isolates of *Nocardiopsis*, 12 showed predatory behavior, out of which 5 preyed on Gram-
292 negative bacteria while 11 preyed on Gram-positive bacteria. Both the isolates of
293 *Micromonospora* preyed on Gram-positive prey while only one preyed on Gram-negative prey.
294 Isolates belonging to genera *Brevibacterium*, *Glutamicibacter* and *Rhodococcus* preyed only
295 on Gram-negative prey while *Rothia* preyed only on Gram-positive prey.
296

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297 **Antibiosis, antibacterial activity and growth inhibition.** Of the 49 actinobacterial isolates
298 screened for antibacterial activity, 25 showed antibiosis against at least one target organism
299 (Supplementary Table S2). Of these 25 isolates, all showed antibiosis against at least one of
300 the Gram-positive target species, while only five showed antibiosis against at least one of the
301 Gram-negative organisms. The frequency of antibacterial activity against Gram-positive
302 organisms was significantly higher (Mann-Whitney U = 1.5, P = 0.003) than those against
303 Gram-negative organisms (Table 2). Most antibacterial activities were broad spectrum with
304 respect to the target organisms that they affected. There were 10 actinobacterial isolates that
305 showed antibiosis against two target organisms, 6 isolates that affected 4 target species and 2
306 isolates that affected 6 target species. There was no association between antibacterial activity
307 and the source (sponge or associated environment) of the isolation ($\chi^2 = 2.543$, P = 0.111).

308 Out of eight isolates of *Streptomyces* that were screened for antibacterial activity, five
309 showed antibiosis, of which two showed antibiosis against Gram-negative target species, while
310 all showed antibiosis against Gram-positive organisms. In the case of *Nocardiopsis*, of the 25
311 isolates used for screening 17 showed antibiosis, of which all affected growth of Gram-positive
312 organisms, while only two affected growth of Gram-negative organisms. Genus *Kytococcus*
313 showed antibiosis that affected both Gram-positive as well as Gram-negative organisms, while
314 *Glutamicibacter* and *Rothia* showed antibiosis against Gram-positive organisms only.

315

316 **Enzyme inhibition.** Out of 49 actinobacterial isolates screened for inhibition of four enzymes,
317 30 isolates inhibited at least one of the enzyme (Supplementary Table S2). Of these 30 isolates,
318 28 inhibited trypsin, 24 inhibited chymotrypsin, three inhibited angiotensin converting enzyme
319 (ACE) and only two inhibited subtilisin. Venn diagram of frequency of isolates inhibiting
320 different enzymes (Figure 3) suggested that five isolates inhibited only trypsin and one isolate
321 each inhibited chymotrypsin and ACE, while subtilisin inhibition was accompanied by

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322 inhibition of other enzymes. No isolate inhibited all four enzymes. Out of 30 actinobacteria
323 that produced enzyme inhibitors, 19 produced two inhibitors, four produced three inhibitors
324 while seven produced only one of the four inhibitors. There was no association between the
325 enzyme inhibition and source of the actinobacterial isolate (χ^2 = 2.2252, P = 0.1358).

326 Out of eight isolates of *Streptomyces* seven produced enzyme inhibitors against
327 proteases, while 12 out of 25 isolates of *Nocardiopsis* produced enzyme inhibitors of which 11
328 produced against proteases and two produced against ACE (Table 3). One isolate of
329 *Actinomycetospora* inhibited activity of ACE.

330

331 **Associations between different activities.** Out of 49 actinobacterial isolates that were
332 screened for activities, 39 showed at least one of the three activities. Of these 39 isolates, 15
333 showed all three activities, while nine showed predation as well as enzyme inhibition (Figure
334 4). There were only seven isolates that showed predation and antibiotic production against the
335 same target organism (Table 2) and all these isolates belonged to genera *Streptomyces* and
336 *Nocardiopsis*.

337 Antibiotic production showed no significant association with predation (χ^2 = 2.4522, P
338 = 0.11736) or any of the four enzyme inhibition (χ^2 = 0.98702, P = 0.32047). However, there
339 were significant associations between predation and protease inhibitors (Figure 5). There were
340 24 isolates that showed both predation as well as inhibition of at least one enzyme and there
341 was a significant association between the two activities (χ^2 = 22.543, P < 0.0001), where
342 predators proportionately produced more enzyme inhibitors than non-predators (Figure 5a).
343 There were 23 actinobacterial isolates that showed predation as well as trypsin inhibition and
344 there was a significant association between the two (χ^2 = 22.185, P < 0.0001) with predators
345 more likely to produce trypsin inhibitors than non-predators (Figure 5b). Similarly, 24
346 actinobacteria were predators as well as inhibited chymotrypsin activity and there was a

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347 significant association between the two (χ^2 = 41.612, P < 0.0001) with predators more likely
348 to produce chymotrypsin inhibitors than non-predators (Figure 5c).
349

350 **DISCUSSION**

351 Sponges and associated environment in northern parts of western coast of India are rich in
352 actinobacterial diversity with about 60 putative species under 16 families and 25 genera. We
353 recorded 11 species of actinobacteria only from sponges. Out of these, *Mycobacterium*
354 *poriferae* was originally described from marine sponge (Padgitt and Moshier, 1987), while
355 three species, *Gordonia terrae* (Elfalah et al., 2013; Santos et al., 2019; Montalvo et al., 2005),
356 *Brevibacterium casei* (Kiran et al., 2010) and *Kocuria rhizophila* (Palomo et al., 2013), have
357 been previously reported from sponges. To our knowledge, we provide first report of species,
358 namely *Brachybacterium murisi, Nocardiopsis salina*, *Pseudonocardia kongjuensis*,
359 *Rhodococcus zopfii*, *Rothia terrae*, *Streptomyces smyrnaeus* and *Streptomyces viridobrunneus*,
360 from marine sponges, although some of them are known from marine habitats (Stach et al.,
361 2003; Satheeja and Jebakumar, 2011).

362 *Streptomyces* was the most dominant genus among the isolates, which agrees with the
363 findings of Zhang et al. (2008). Genus *Nocardiposis*, with its two species *N. alba* and *N.*
364 *dassonvillei*, has been suggested (Bennur et al., 2015) as the second common genus after
365 *Streptomyces* and that too agrees with our findings. Further, report of most genera, including
366 *Agrococcus*, *Arthrobacter*, *Brevibacterium*, *Kocuria*, *Microbacterium* and *Micrococcus*, from
367 sponges in our study are consistent with previous reports from other study areas including
368 South China Sea (Li et al., 2015), Yellow Sea (Zhang et al., 2008), Mediterranean Sea (Cheng
369 et al., 2015), coast of Florida in USA (Montalvo, 2005) and northern coast of Brazil (Menezes
370 et al., 2010) indicating that there are common trends in the discovery of actinobacteria from
371 sponges.

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372 Among the first reports from marine environment from our study, *Aeromicrobium*
373 *massiliense* and *Glutamicibacter mysorens* are known from human fecal microbiota
374 (Ramasamy et al., 2012) and sewage (Nand and Rao, 1972) respectively. Presence of these two
375 species in the sediments along the collection site Harne (17.81ºN, 73.09ºE) likely suggests fecal
376 pollution in this area.

377 Although predation is a widespread behavior in bacterial kingdom, δ-proteobacteria of
378 the orders *Myxococcales* and *Bdellovibrionales* have received more attention (Jurkevitch,
379 2007) as compared to other taxa, especially the Gram-positive bacteria such as actinobacteria.
380 Among actinobacteria only three genera, namely *Agromyces*, *Streptomyces* and
381 *Streptoverticillium*, are known to have predatory behavior against other bacterial species
382 (Casida, 1980; 1983; 1988; Kumbhar et al., 2014; Zeph and Casida, 1986). In the current study,
383 for the first time, we show predation in six other genera of actinobacteria, namely
384 *Brevibacterium*, *Glutamicibacter*, *Micromonospora*, *Nocardiopsis*, *Rhodococcus* and *Rothia*.
385 Kumbhar et al. (2014) argued that predatory behavior is widespread in genus *Streptomyces* and
386 even in the current study we observed that all the isolates of *Streptomyces* used for screening
387 showed predation on Gram-positive as well as Gram-negative prey.

388 Since sponges are sessile and lack other anti-predator defenses, it has been suggested
389 that secondary metabolites of bacteria can provide sponges with chemical defense (Lee et al.,
390 2001; Kumbhar and Watve, 2013). However, we did not observe any significant association
391 between the source of actinobacterial isolation and antibiotic production, suggesting that
392 isolates even from environment were equally likely to produce antimicrobials as that of the
393 isolates recovered from sponges. However, there was a significant association between the
394 source of isolation and predatory activity, with proportionately more predators among the
395 isolates recovered from sponge. Ecologically this makes sense. As the sponges are filter feeders
396 and have regular intake of environmental bacteria, sponge associated actinobacteria will have

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397 better predation opportunities. It is also possible that the predatory activity of sponge associated
398 actinobacteria, could have evolved as a mutualistic activity as it can defend sponges from
399 pathogenic bacterial invasions.

400 Actinobacteria are known to produce several enzyme inhibitors (Manivasagan et al.,
401 2015; Imada, 2005). However, for the first time we show a strong association between
402 predation and enzyme inhibition, specifically inhibition of trypsin and chymotrypsin, where
403 predators produced proportionality more enzyme inhibitors as compared to non predators.
404 Predators themselves are known to produce a variety of hydrolytic enzymes for degrading the
405 prey (Pérez et al., 2016). Therefore, it is possible that the production of enzyme inhibitors
406 safeguards their own cells from being target of the enzyme. It is also possible that enzyme
407 inhibitors also protect the actinobacteria from hydrolytic enzymes produced from the sponge
408 host and other microbiota.

409 An interesting observation that we made, when comparing the predation and antibiotic
410 production by actinobacteria, was that, while predation was equally effective against Gram-
411 positive as well as Gram-negative target species, antibiotic production was mainly effective
412 against Gram-positive bacteria. It is therefore possible that studying the predatory behavior of
413 actinobacteria and predation specific metabolites could lead to discovery of novel therapeutic
414 agents that are more broad-spectrum.

415 Although actinobacteria are known to be rich in secondary metabolites, extracellular
416 enzymes and enzyme inhibitors, the ecological role of these extracellular bioactive molecules
417 is little known. We suggest that studying the ecological correlates of bioactivity and the inter-
418 correlation patterns of different types of bioactivity can be a useful tool in understanding the
419 ecological origins of bioactivity and testing alternative ecological hypotheses.

420

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422 **CONCLUSION**

423 Sponges and associated environments of intertidal zones, along the northern parts of west coast
424 of India, are rich in actinobacterial diversity with 16 families and 25 genera, which could be
425 attributed to 55 putative species using PTP and 60 putative species based on bPTP methods.
426 Although, at the genus level, the trends in the discovery of actinobacteria isolated from sponges
427 was consistent with previous studies from different study areas, we provide first report of six
428 species, namely *Brachybacterium murisi, Nocardiopsis salina*, *Pseudonocardia kongjuensis*,
429 *Rhodococcus zopfii*, *Rothia terrae*, *Streptomyces smyrnaeus* and *Streptomyces*
430 *viridobrunneus*,. Non-obligate epibiotic predatory behavior was widespread among
431 actinobacterial genera and we provide first report of predatory activity in *Brevibacterium*,
432 *Glutamicibacter*, *Micromonospora*, *Nocardiopsis*, *Rhodococcus* and *Rothia*. Sponges
433 associated actinobacteria showed significantly more predatory behavior than environmental
434 isolates, and we hypothesize that predatory actinobacteria might provide sponges with defense
435 against pathogenic bacteria. While antibiotic produced from actinobacterial isolates affected
436 Gram-positive target bacteria with little to no effect on Gram-negative bacteria, predation
437 targeted both Gram-positive and Gram-negative prey with equal propensity, suggesting that
438 study of predation specific metabolites might provide novel therapeutic agents with broad-
439 spectrum. Actinobacterial isolates from both sponge and associated environment produced
440 inhibitors of serine proteases and angiotensin converting enzyme. Predatory behavior was
441 strongly associated with inhibition of trypsin and chymotrypsin, which might be helpful for the
442 actinobacteria for overcoming effects of proteolytic enzymes produced by sponge host and
443 other microbiota. Understanding diversity and associations among various actinobacterial
444 activities, with each other and the source of isolation, can provide new insights in marine
445 microbial ecology and provide opportunities to isolate novel therapeutic agents.

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448 **DATA AVAILABILITY**

449 Sequences of 16S rRNA gene of studied isolates are submitted to GenBank NCBI under the
450 accession numbers MN339687–MN339897. All the data used for analysis is provided in
451 supplementary information.

452

453 **ACKNOWLEDGEMENTS**

454 This work was funded by Maharashtra Gene Bank Programme (RGSTC/File-2007/DPP-
455 054/CR-28) of Rajiv Gandhi Science and Technology Commission, Government of
456 Maharashtra, India. We thank the Director and Chair of Biology, Indian Institute of Science
457 Education and Research (IISER), Pune, and Principle, M.E.S. Abasaheb Garware College,
458 Pune for providing infrastructural facilities. We are thankful to V. S. Rao, IISER Pune, for
459 support an encouragement.

460

461 **AUTHOR CONTRIBUTIONS**

462 M.W., U.B. and N. Deshpande conceived and designed the study. U.B., N.S., K.H., A.P., U.L.,
463 T.G., K.P., A.J., R.S., H.V and V.T. performed the study. N. Dahanukar and M.W. analyzed
464 the data. N. Dahanukar, U.B. and M.W. wrote the manuscript with inputs from other authors.
465 All authors contributed to the proofreading of the manuscript.

466

467 **Additional information**

468 Supplementary information: Supplementary Table S1 and Table S2 accompanies the online
469 version of the paper.

470 Competing Interests: The authors declare no competing interests.

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665 **Table 1.** Putative number of species of actinobacterial genera based on PTP and bPTP methods

666 isolated from sponge, associate environment and both sources.

667

Family	Genus	Sponge		Environment		Both	
		PTP	bPTP	PTP	bPTP	PTP	bPTP
Actinomycetaceae	*Streptomyces*	7	8	8	9	4	5
Brevibacteriaceae	*Brevibacterium*	5	6	2	2	1	1
Cellulomonadaceae	*Cellulomonas*	0	0	1	1	0	0
Dermabacteraceae	*Brachybacterium*	1	1	2	2	0	0
Dietziaceae	*Dietzia*	0	0	1	1	0	0
Geodermatophilaceae	*Klenkia*	0	0	1	1	0	0
Gordoniaceae	*Gordonia*	1	1	0	0	0	0
Intrasporangiaceae	*Janibacter*	0	0	2	2	0	0
	Knoellia	0	0	1	1	0	0
	Kytococcus	0	0	1	1	0	0
Microbacteriaceae	*Agrococcus*	1	1	1	1	1	1
	Curtobacterium	0	0	1	1	0	0
	Microbacterium	1	1	1	2	1	1
Micrococcaceae	*Arthrobacter*	1	1	2	2	1	1
	Glutamicibacter	0	0	1	1	0	0
	Kocuria	3	4	4	4	2	1
	Micrococcus	1	1	2	2	1	1
	Rothia	1	1	0	0	0	0
Micromonosporaceae	*Micromonospora*	1	1	1	1	1	1
Mycobacteriaceae	*Mycolicibacterium*	1	1	0	0	0	0
Nocardiaceae	*Rhodococcus*	2	2	2	2	1	1
Nocardioidaceae	*Aeromicrobium*	0	0	1	1	0	0
Nocardiopsaceae	*Nocardiopsis*	3	3	3	3	2	2
Pseudonocardiaceae	*Actinomycetospora*	0	0	2	2	0	0
	Pseudonocardia	1	1	0	0	0	0
	Total	30	33	40	42	15	15

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671 **Table 2.** Predation and antibiotic production by actinobacteria against the Gram positive and

672 Gram negative target species.

673

Target species	Predation	Antibiotic	Predation and Antibiotic by same actinobacterial isolate
Gram positive			
Mycobacterium smegmatis	3	12	0
Micrococcus luteus	8	5	0
Bacillus subtilis	1	24	1
Staphylococcus aureus	17	9	4
Salinicoccus roseus	9	3	0
Enterococcus faecalis	3	20	1
Gram negative			
Acetobacter pasterianus	7	0	0
Alcaligenes faecalis	3	1	1
Escherichia coli	2	5	0
Klebsiella pneumoniae	3	0	0
Proteus vulgaris	8	0	0
Salmonella enterica	2	0	0
Serratia marcescens	3	0	0
Pseudomonas aeruginosa	1	0	0

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676 **Table 3.** Frequency of actinobacterial isolates producing four different enzyme inhibitors.

677

Genus	Number of isolates	Frequency of isolates inhibiting				Isolates with at least one inhibition activity
		Subtilisin	Trypsin	Chymotrypsin	ACE	
Actinomycetospora	2	0	1	0	1	2
Agrococcus	1	0	0	0	0	0
Brevibacterium	1	0	1	1	0	1
Glutamicibacter	1	0	1	1	0	1
Kocuria	1	0	0	0	0	0
Kytococcus	1	0	1	0	0	1
Micrococcus	1	0	1	0	0	1
Micromonospora	2	0	2	2	0	2
Nocardiopsis	25	0	11	11	2	12
Pseudonocardia	1	0	0	0	0	0
Rhodococcus	4	0	2	1	0	2
Rothia	1	0	1	1	0	1
Streptomyces	8	2	7	7	0	7

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680 **Figure 1.** Maximum likelihood phylogenetic tree of actinobacterial isolates based on TIM3+R6

681 nucleotide substitution model. Firmicutes belonging to genus *Bacillus* were used as outgroups.

682 Sequence codes in red were used for screening.

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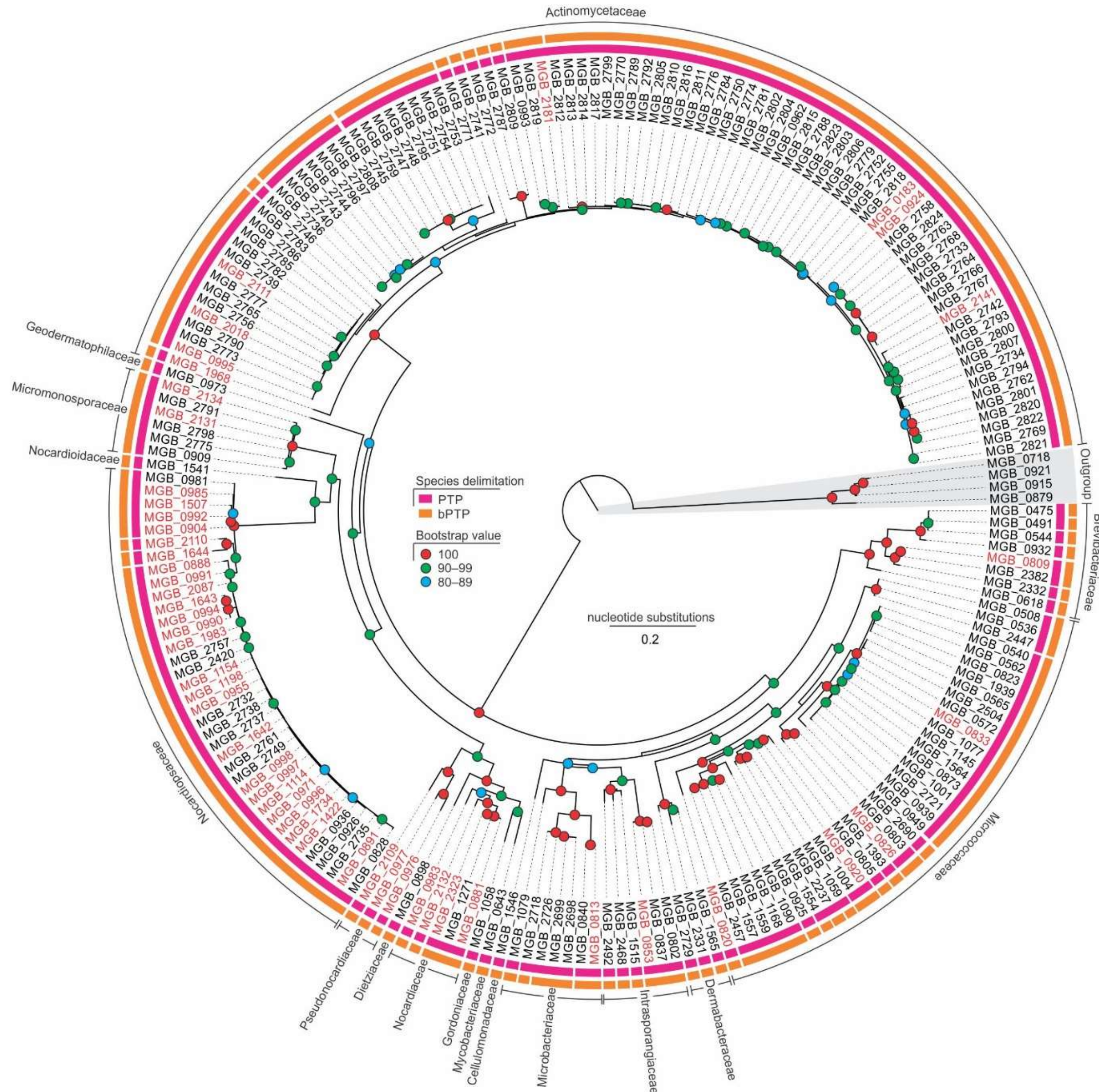

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686 **Figure 2.** Association between source of actinobacterial isolation on their predatory behavior.
687 There was a significant association between the source (sponge or associated environment) of
688 actinobacterial isolation and predation (χ^2 = 6.200, P = 0.0128).
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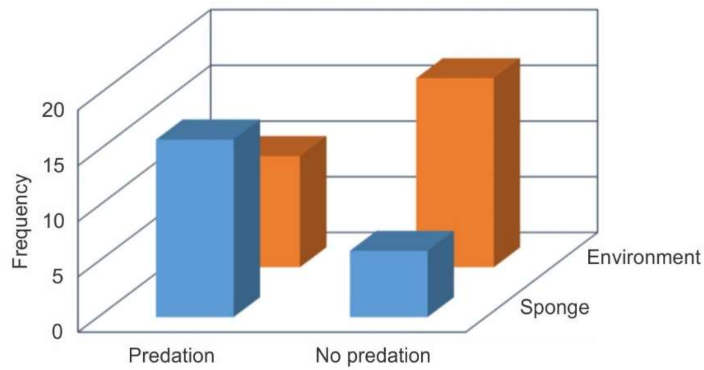

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693 **Figure 3.** Venn diagrams combination of enzyme inhibitors produced by actinobacterial
694 isolates. Venn diagrams is not to scale.
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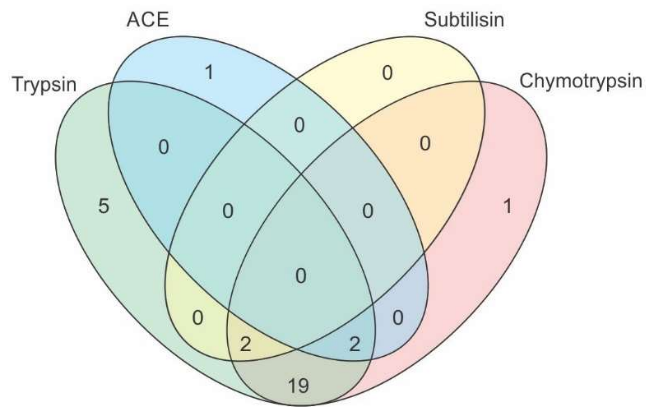

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699 **Figure 4.** Venn diagrams of predation, antibiotic production and enzyme inhibition by
700 actinobacterial isolates. Venn diagrams is not to scale.
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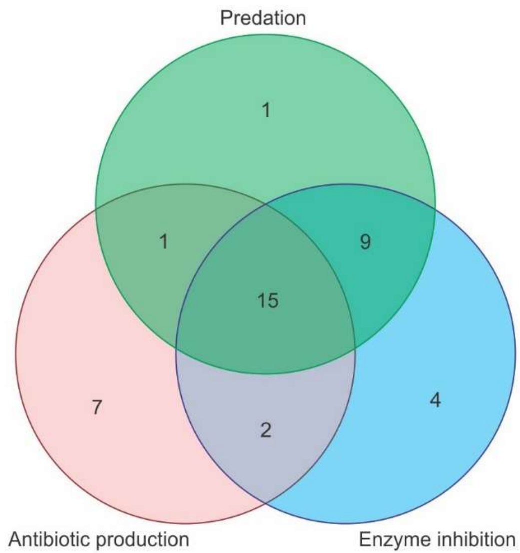

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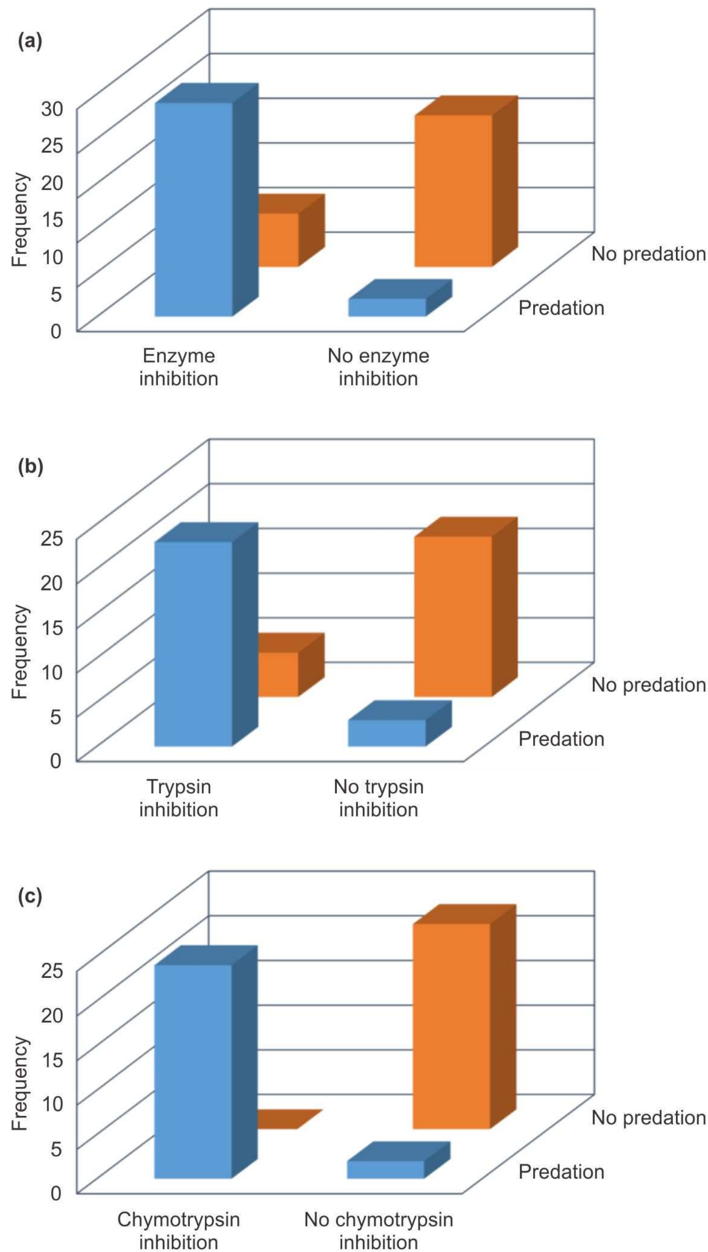
704 **Figure 5.** Association between enzyme inhibition and predation in actinobacterial isolates.
705 Predation was significantly associated with (a) inhibition of any one of the four enzymes tested
706 ($\chi^2 = 22.543$, $P < 0.0001$), (b) inhibition of trypsin ($\chi^2 = 22.185$, $P < 0.0001$) and (c) inhibition
707 of chymotrypsin ($\chi^2 = 41.612$, $P < 0.0001$).
708



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