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BRIEF REPORT

ENVIRONMENTAL MICROBIOLOGY

Unveiling the culturable and non-culturable actinobacterial diversity in two macroalgae species from the northern Portuguese coast

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Abstract

Actinomycetota, associated with macroalgae, remains one of the least explored marine niches. The secondary metabolism of Actinomycetota, the primary microbial source of compounds relevant to biotechnology, continues to drive research into the distribution, dynamics, and metabolome of these microorganisms. In this study, we employed a combination of traditional cultivation and metagenomic analysis to investigate the diversity of Actinomycetota in two native macroalgae species from the Portuguese coast. We obtained and taxonomically identified a collection of 380 strains, which were distributed across 12 orders, 15 families, and 25 genera affiliated with the Actinomycetia class, with Streptomyces making up approximately 60% of the composition. Metagenomic results revealed the presence of Actinomycetota in both *Chondrus crispus* and *Codium tomentosum* datasets, with relative abundances of 11% and 2%, respectively. This approach identified 12 orders, 16 families, and 17 genera affiliated with Actinomycetota, with minimal overlap with the cultivation results. Acidimicrobiales emerged as the dominant actinobacterial order in both macroalgae, although no strain affiliated with this taxonomic group was successfully isolated. Our findings suggest that macroalgae represent a hotspot for Actinomycetota. The synergistic use of both culture-dependent and independent approaches proved beneficial, enabling the identification and recovery of not only abundant but also rare taxonomic members.

INTRODUCTION

Actinomycetota represents one of the major Bacteria phyla, both in number and diversity (Barka et al., 2016). Members of this phylum can be found in sundry environments, including marine ecosystems where they can establish symbiotic interactions with other organisms, as macroalgae (Girão et al., 2019). One of the most recognizable traits of these microorganisms is their unrivalled ability to synthesize biotechnologically relevant natural products (NP) (Girão et al., 2022; van Bergeijk et al., 2020), making the exploration of their metabolism for chemical novelty the driving force of many research programs. This biosynthetic capability is especially noteworthy for a more restricted group of Actinomycetota members, usually designated as actinomycetes. Yet, one major bottleneck in NP research is the limited accessibility to novel sources for exploration (Wright, 2019), which highlights the importance of retrieving novel marine microbes and exploring the extensive pool of underexplored assets that their metabolism encodes (Bull et al., 2005). Macroalgae, also referred to as seaweeds, represent a diverse group of ecological, biotechnological and industrially

significant organisms that can be found in several marine habitats, from intertidal zones to deeper oceanic regions (Abdel-Kareem & ElSaied, 2022; Ortega et al., 2019). They play a major role in nutrient cycling, oxygen production, carbon fixation and coastal protection, with many living beings relying on them as a primary food source, habitat and shelter, stressing their importance in maintaining marine systems health, resilience and biodiversity (Chan et al., 2006). Apart from their ecological role, macroalgae represent a billion-dollar-sized market (Deepika et al., 2022): these organisms are used in human and animal diets (Afonso et al., 2019; García-Vaquero & Hayes, 2016), applied in agriculture as fertilizers (Hamed et al., 2018), represent a potential source of renewable biofuels and bioenergy (Ghadiryanfar et al., 2016) and can synthesize a wide range of biotechnologically-relevant bioactive compounds (Barbosa et al., 2014; Cikoš et al., 2022; Cornish & Garbary, 2010; Olasehinde et al., 2019; Pimentel et al., 2018). Macroalgae host and provide habitat for a wide range of microorganisms, from fungi to bacteria. These microbes form complex and dynamic communities that contribute to the overall health and functioning of the macroalgae, as well as to their interactions with the surrounding environment (Egan et al., 2013). Previous studies have described Actinomycetota associated with species belonging to the phyla Chlorophyta and Rhodophyta and the class Phaeophyceae (green, red and brown, respectively), mostly distributed across Atlantic, Pacific and Baltic Sea Coasts (Braña et al., 2015; Braña et al., 2019; Girão et al., 2019; Goecke et al., 2013; Kim et al., 2018; Wiese et al., 2009), Asia-Pacific (Cho et al., 2012; Cho & Kim, 2012; Kanagasabhapathy et al., 2008; Matsuo et al., 2011; Nguyen et al., 2021; Satheeja Santhi et al., 2014; Ulfah et al., 2017; Uzair et al., 2018; Villarreal-Gómez et al., 2010; Wang et al., 2017) and Antarctica (Alvarado et al., 2018) regions, with many of them exhibiting remarkable bioactivities.

In the Atlantic Ocean, the rugged northern Portuguese coastline features diverse intertidal zones with rocky outcrops and tidal pools that provide habitats for numerous species of macroalgae (Gaspar et al., 2019). *Chondrus crispus* and *Codium tomentosum* represent two native and abundant species from the intertidal Portuguese coast, belonging to the phyla Rhodophyta and Chlorophyta (red and green macroalgae, respectively). Both species can be found attached to rocks and other substrates, thriving in highly dynamic habitats with fluctuating environmental and biological conditions: intertidal rocky areas are subject to various stressing factors such as desiccation, temperature fluctuations, exposure to UV radiation, salinity and osmotic changes, competition for space and resources, grazing and anthropogenic pressure (D'Archino & Piazzi, 2021; Dayton, 1971; Quigley et al., 2020; Rothäusler et al., 2022). Microorganisms able to flourish in association with macroalgae living in such distinctive and challenging ecological niches are expected to possess unique adaptive mechanisms and diversity. Recently, studies using Chlorophyta *Ulva* species have shown that the macroalgae-associated bacterial community, including Actinomycetota, can have beneficial and adverse effects on host growth and development depending on environmental stress conditions (Ghaderiardakani et al., 2017; Hmani et al., 2023). In the single study harnessing exclusively the diversity and bioactive potential of the Actinomycetota culturable community associated with kelp from the northern Portuguese shore, a rich reservoir of taxonomically diverse strains producing antimicrobial and anticancer metabolites was uncovered (Girão et al., 2019). To our knowledge, the Actinomycetota community living in association with *Chondrus crispus* and *Codium tomentosum* has never been explored before, though previous studies have described other bacterial phyla associated with *C. crispus* collected in the same region, namely Planctomycetota (Bondoso et al., 2014), with brief mentions to the presence of Actinomycetota (Picon et al., 2021). In the present study, we aim to survey the Actinomycetota communities living in association with these macroalgae species. By combining metagenomic sequencing and microbial cultivation techniques, as culture-independent and dependent approaches, we targeted to explore the abundance, diversity and taxonomic distribution of this phylum in this symbiotic niche. The main taxa recovered using both methodologies are described and compared, enriching the understanding of the broader landscape of macroalgae-associated actinobacterial communities.

EXPERIMENTAL PROCEDURE

Macroalgae sampling

One specimen of *Chondrus crispus* and one specimen of *Codium tomentosum* were collected in early January 2020 in the intertidal area of the northern Portuguese rocky shore (41.309298°; −8.742228°). The macroalgae were transported to the laboratory under refrigeration conditions and processed on the same day for the study of Actinomycetota culturable and non-culturable communities.

Actinomycetota isolation

The collected macroalgae (one specimen per species) were thoroughly washed with sterile seawater, to remove any larger particles attached, and each dissected into two distinct parts: holdfast and blades. Each part was segmented into smaller pieces and macerated until the obtainment of 0.5 g of macerated tissues

(in total, each macroalga yielded two macerated tissue samples, extracted from both its holdfast and blades, totalling four samples altogether). To increase the success of Actinomycetota isolation, by limiting the incidence of non-spore-forming microorganisms and potentiating the development of slow-growing strains, the four samples of macerated tissues were separately placed in 2 mL tubes containing 1 mL of sterile seawater and incubated in a water bath at 58°C for 15 min. The resulting samples were 10-fold diluted until 10^{-2}, using sterile seawater, and 100 μL of each dilution was inoculated in duplicate in four distinct selective culture media for Actinomycetota, to enhance the recovery of strains with different growth requirements and metabolic profiles: Nutrient-poor Sediment Extract agar (NPS), Actinomycete Isolation Agar (AIA), Starch-Casein-Nitrate agar (SCN) and Seaweed Agar (SA) (Table 1). SA was formulated to mimic the natural environment of macroalgae-associated microbial symbionts, being solely composed of the tissues of each macroalgae reduced to powder using liquid nitrogen and seawater. Pieces of the macerated tissues (one 10 μL loop) were also directly inoculated, in duplicate, on the four agar media mentioned above. The 98 generated plates were incubated for a period of up to 6 months at room temperature (±24°C) and periodically inspected to track bacterial growth. All morphologically distinct colonies were streaked on the respective isolation medium until obtainment of pure cultures and preserved at −80°C in 30% (v/v) glycerol.

Identification of the isolates and phylogenetic analysis

All isolates were taxonomically identified through 16S rRNA gene sequencing. Biomass for DNA extraction was obtained by growing each isolate at 28°C, for 1 week, in the corresponding isolation agar media (Table S1). Genomic DNA was extracted using the E.Z.N.A. Bacterial DNA Kit (Omega Bio-Tek, GA, United States) according to the manufacturer's instructions. The 16S rRNA gene was amplified by PCR using the universal primers 27F/1492R (Weisburg et al., 1991), as described by Girão et al. (2019). The acquired 16S rRNA gene sequences were analysed using Geneious Prime 2023.1 software (Biomatters, Auckland, New Zealand). EzTaxon tool from the EzBioCloud database (Chun et al., 2007) was used to establish strain taxonomic affiliation based on consensus sequence similarity with deposited quality-controlled 16S rRNA data. All sequences were deposited in GenBank (NCBI, Bethesda, MD, USA) (Table S1). A phylogenetic study was performed to in-depth understanding of the influence of the isolation origin (*C. tomentosum* or *C. crispus* tissues) on the evolutionary relationship

TABLE 1 Formulation of the selective culture media used for the isolation of Actinomycetota.

NPS[a]		**AIA**[b]	
Agar	17 g	Agar	17 g
Marine sediment extract (obtained by washing beach sand with 500 mL of seawater)	100 mL	Sodium propionate	4 g
		K_2HPO_4	
		Na_2CO_3	0.5 g
		$FeSO_4$	0.2 g
		L-arginin	0.2 g
		$MgSO_4$	0.1 g
			0.2 g
SCN[b]		**SA**[a]	
Agar	17 g	Agar	17 g
Soluble starch	10 g	Seaweed powder	10 g
Casein	0.3 g		
K_2HPO_4	2 g		
KNO_3	2 g		
NaCl	2 g		
$MgSO_4.7H_2O$	0.05 g		
$CaCO_3$	0.02 g		
$FeSO_4.7H_20$	0.01 g		

Note: All media were supplemented with cycloheximide (50 mg L^{-1}), nalidixic acid (50 mg L^{-1}) and nystatin (50 mg L^{-1}) (Sigma-Aldrich, MO, United States).
[a]Per litre of seawater.
[b]Per litre of 60:40 seawater/deionized water.

between the retrieved Actinomycetota strains. Two phylogenetic trees, one comprising all strains identified as *Streptomyces* and the other all the non-*Streptomyces* isolates (Figures 1 and 2, respectively) were constructed. The sequences were aligned using MUSCLE from within the Geneious software package and the Maximum Likelihood method with 1000 bootstraps based on the Tamura-Nei model applied. MEGA-X (Kumar et al., 2018) was used to build the tree and iTOL to perform its final display and annotation (Letunic & Bork, 2019). Strains identified as potential novel species were subjected to an individual phylogenetic analysis as well. According to EzTaxon database results, for the taxonomic study of each potential novel species, the 15 closest related valid species were selected, with no more than a single sequence being selected for the same species, and a phylogenic tree was constructed as described above.

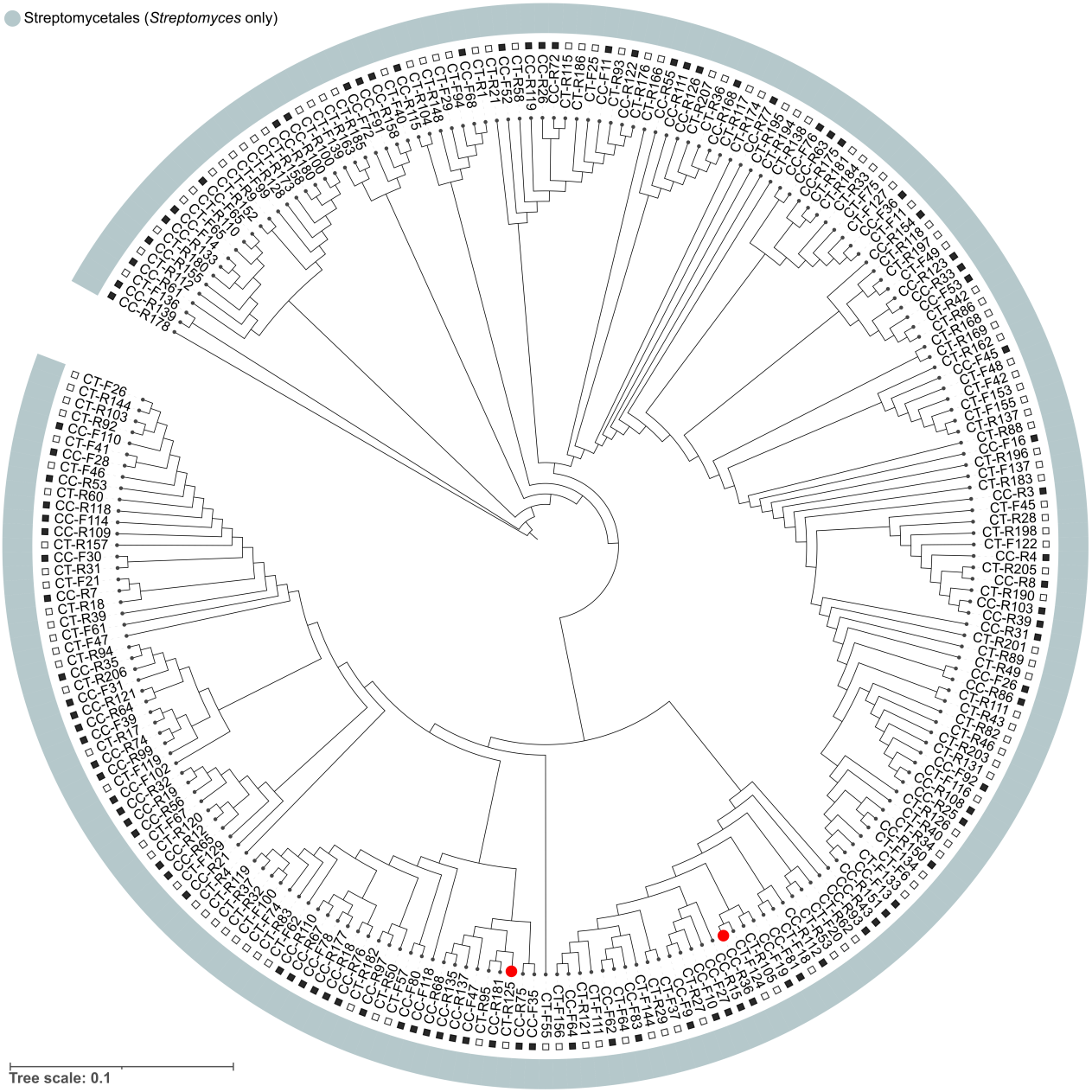

FIGURE 1 Phylogenetic tree based on the 16S rRNA gene, obtained by Maximum Likelihood analysis of Actinomycetota isolates recovered from *C. crispus* (■) and *C. tomentosum* (□) affiliated to the genus *Streptomyces*. Genomic data from strains CT-R87 and CT-F145 were not considered due to the small size of their consensus sequences. The tree was generated using an alignment of 1522 bp and 1000 bootstraps. The order-level affiliation of the strains is coloured indicated as described in the picture caption. Strains representing potential novel taxa (●) are pointed out as well.

FIGURE 2 Phylogenetic tree based on the 16S rRNA gene, obtained by Maximum Likelihood analysis of Actinomycetota isolates recovered from *C. crispus* (■) and *C. tomentosum* (□) affiliated to non-*Streptomyces* genera. Genomic data from strains CC-F55 and CT-R135 were not considered due to the small size of their consensus sequences. The tree was generated using an alignment of 1345 bp and 1000 bootstraps. The order-level affiliation of the strains is coloured indicated as described in the picture caption. Strains representing potential novel taxa (●) are pointed out as well.

DNA extraction and quantification for metagenomic analysis

For each macroalgae species, 2 g of tissue was macerated with the aid of liquid nitrogen and placed in a collection tube. Environmental DNA (eDNA) was extracted using DNeasy PowerSoil Pro Kit (QIAGEN, Inc., Germany), according to the manufacturer's instructions. The obtained DNA was quantified using a Qubit Fluorometer (Thermo Fisher Scientific—United States).

Metagenomic sequencing and analysis

For shotgun metagenomic sequencing, libraries were first generated using Illumina TruSeq Nano DNA LT Library Preparation Kit and index codes were added to attribute sequences to each sample. Briefly, for each sample, DNA was fragmented by sonication to a size of ~400 bp, and then fragments were end-polished, A-tailed and ligated with the full-length adaptor for Illumina® sequencing with further PCR amplification. The PCR amplification aimed to selectively enrich DNA

fragments that had been ligated with the full-length adaptor (R1: AATGATACGGCGACCACCGAGATCT ACAC, R2: TAGAGCATACGGCAGAAGACGAAC). Finally, PCR products were purified (AMPure XP system) and libraries were analysed for size distribution by Agilent2100 Bioanalyzer and quantified using real-time PCR. Paired-end reads were generated in an Illumina NovaSeq platform (Illumina, USA) with a PE150 platform. All taxonomical inferences produced from these metagenomic datasets were performed in KBase (Arkin et al., 2018). For this, paired-end libraries from each sample were first merged and low-quality reads (<36 bp, <Q15 within 4-base windows) were subtracted from the datasets using Trimmomatic (Bolger et al., 2014). Then, read hygiene analysis of the post-trimmed libraries was performed using the FastQC function (Andrews, 2010) to confirm the quality of these libraries for the downstream annotations. To trace the profiles of prokaryotic taxonomy for each macroalgae, unassembled high-quality metagenomic reads were taxonomically annotated using the Kaiju taxonomic classifier v1.9.0 (Menzel et al., 2016) using the RefSeq Genome database (not including eukaryotes) as reference. Database searches were performed for all taxonomic ranks in the Maximum Exact Matches mode, with no sub-sampling and considering a relative abundance threshold of 0.1%.

RESULTS AND DISCUSSION

Isolation, diversity and phylogeny of culturable macroalgae-associated Actinomycetota

To investigate the diversity of culturable Actinomycetota associated with the macroalgae *C. tomentosum* and *C. crispus*, specimens of these organisms were collected in the intertidal northern Portuguese rocky shore and processed for bacterial isolation. From the two collected macroalgae, a total of 380 Actinomycetota strains were isolated into pure cultures (210 from *C. tomentosum* tissues and 170 from *C. crispus*), and taxonomically identified. A diverse array of typically morphological and physiological traits associated with this phylum was observed within the collection, with strains encompassing a wide range of sizes, shapes and colours, production of mycelial networks, spores and pigments, synthesis of volatile compounds translated in a distinctive earthy odour and overall slow growth (Figure 3A–P). To our knowledge, these 380 strains represent the largest published collection of macroalgae-associated Actinomycetota to date.

To better explore if any particular region of the macroalgae was more prolific for Actinomycetota isolation, the collected specimens were segmented into two parts: holdfast and blades. Several strains were recovered from both regions, with a higher number of isolates being retrieved from holdfasts in both specimens. These findings are consistent with previous results for the kelp *Laminaria ochroleuca* (Girão et al., 2019), emphasizing the prevalence of this taxa in this part of the thallus, likely due to the micro-environments established by distinct morphological niches in the host (Morrissey et al., 2019). Four different culture media—distinctive in nutrient composition—were used in this work to isolate the target microorganisms, with more strains being obtained with the medium AIA (Figure 4A, Table S1). Former studies have also used this medium to successfully retrieve Actinomycetota from diverse origins, including marine sources (Abdelmohsen et al., 2010; Valli et al., 2012; Zainal Abidin et al., 2016). The oligotrophic medium, NPS, was used to replicate the nutrient-poor conditions typically found in the marine environment, while the tailor-made medium, SA, was employed to simulate the conditions that the macroalgae under investigation offer to their symbiotic partners. By granting limited and selective nutritional sources, these media can support the growth of bacteria more adapted to marine environments and, more specifically, of those living in symbiotic relationships with macroalgae. Conversely, AIA and SCN, richer in carbon and nitrogen sources, suit better strains that require higher nutritional standards to thrive. The fact that all culture media were effective in the isolation of the target taxa, pinpoints dissimilar metabolic profiles and requirements among the obtained bacteria. This is interesting not only from a diversity angle but also from a biotechnological perspective: metabolic plasticity is greatly relevant concerning the synthesis of unique bioactive secondary metabolites, a distinctive and remarkable actinobacterial trait. Likewise, SA developed in this work, has been shown that supplementing culture media with the host macroalgae might benefit the growth of Actinomycetota and the production of valuable molecules as antibiotics (Okazaki et al., 1975).

To confirm Actinomycetota affiliation and determine taxonomic identity, all 380 isolates were identified through 16S rRNA gene sequencing. According to the genomic data, these strains are distributed across 12 orders, 15 families and 25 genera affiliated with the Actinomycetia class (Figure 4B,C). The genus *Streptomyces* emerged as the prevailing taxonomic group, with 231 strains successfully isolated, representing nearly 61% of all collections, followed by the genera *Rhodococcus*, *Nocardiopsis* and *Micromonospora* with 36, 25 and 22 isolates, respectively. This distribution was observed in both macroalgae, with 131 *Streptomyces* retrieved from *C. tomentosum* and 100 from the tissues of *C. crispus*, followed by the higher abundance of *Rhodococcus*, *Nocardiopsis* and *Micromonospora* strains (Figure 4C). The predominance of *Streptomyces* is not unexpected, since they represent the most abundant culturable Actinomycetota, and are well adapted to the marine environment (Tian et al., 2016). In a previous study harnessing kelp-associated

FIGURE 3 Examples of the morphological diversity of Actinomycetota strains isolated from *C. crispus* and *C. tomentosum* macroalgae: (A) *Streptomyces griseoaurantiacus* CT-R1; (B) Streptomyces sp.CT-F26; (C) Streptomyces griseorubens CC-R18; (D) Streptomyces sp. CC-F30; (E) Streptomyces sp. CC-R86; (F) Micromonospora sp.CC-F88; (G) Streptomyces sp.CT-R29; (H) Micromonospora chalcea CT-F151; (I) Streptomyces sp.CC-R33; (J) *Actinoalloteichus hoggarensis* CC-R176; (K) Streptomyces sp. CT-F61; (L) *Streptomyces griseoaurantiacus* CC-F68; (M) Nocardiopsis alba CC-F77; (N) *Streptomyces griseoaurantiacus* CT-F94; (O) Streptomyces sp. CC-F102; (P) *Streptomyces mayteni* CC-R139.

Actinomycetota from the same environment, the over-representation of this particular taxon was also observed (Girão et al., 2019). Given its high occurrence in the studied macroalgae, we can hypothesize on its significance to the host interaction and surrounding environment. *Streptomyces* are the major microbial source of bioactive secondary metabolites with a broad range of properties (Hutchings et al., 2019), and several isolates belonging to this genus have been isolated from macroalgae and explored for the production of such entities (Xiong et al., 2023). Given their proven ability to produce antimicrobial, antifungal, anti-inflammatory and antifouling compounds, it is reasonable that this wide chemical arsenal might benefit the host, providing a defence to ensure its survival and competitiveness in the environment (Abdul Malik et al., 2020). Nonetheless, it is important to mention that non-*Streptomyces* Actinomycetota have also proven their value as producers of bioactive metabolites (Gavriilidou et al., 2022). While most of the genera were found in both macroalgae, some were obtained only from one of the species: strains affiliated to *Actinoalloteichus*, *Actinomadura*, *Citricoccus*, *Krasilnikoviella*, *Saccharomonospora*, *Stackebrandtia* and

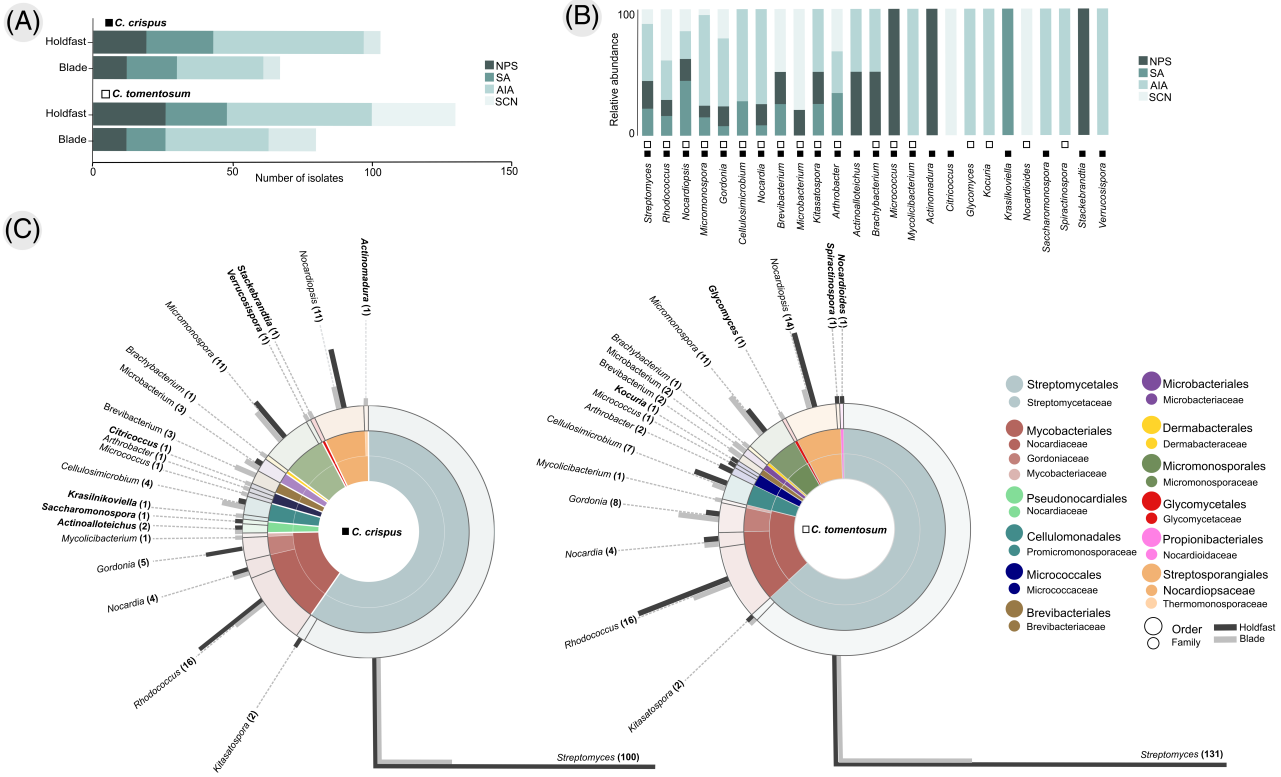

FIGURE 4 Actinomycetota recovered from *C. crispus* (■) and *C. tomentosum* (□). The number of isolates recovered from the holdfast and blade of each macroalgae species, and the respective isolation media (AIA, Actinomycete isolation agar; NPS, nutrient-poor sediment extract agar; SA, seaweed agar; SCN, starch-casein-nitrate agar), are presented (A) as well as the distribution of the genera recovered (B). The taxonomic distribution of the strains—order, family, and genus, from the inside to the outside of the circle, respectively—is presented according to the part of the macroalgae from which they were isolated. Numbers in brackets indicate the number of isolates retrieved from each genus. Genera exclusively obtained from a macroalgae species are highlighted in bold (C).

Verrucosispora were solely isolated from *C. crispus* and strains associated to *Glycomyces*, *Kocuria*, *Nocardioides* and *Spiractinospora* were only recovered from *C. tomentosum*. Actinomycetota strains were isolated both from the holdfast and blades of the macroalgae. While some genera were obtained from both regions, others were solely retrieved from a specific section of the hosts: *Brachybacterium*, *Brevibacterium*, *Citricoccus*, *Glycomyces*, *Kocuria*, *Krasilnikoviella*, *Mycolicibacterium*, *Stackebrandtia* and *Verrucosispora* strains were exclusively isolated from the blades of the macroalgae and *Actinomadura*, *Nocardioides*, *Saccharomonospora* and *Spiractinospora* from the holdfasts. Interestingly, though a higher number of isolates were obtained from the holdfast of the two macroalgae species, the blades proved to be a better area for Actinomycetota diversity (Figure 4C). Linking the isolation culture media with strains diversity, the selective medium AIA was the one associated to a more diverse range of culturable Actinomycetota, enabling the recovery of 17 genera, followed by NPS, SP and NPS media, with 14, 11 and 10 genera recovered, respectively (Figure 4B). The richest media, AIA and SCN, enabled the retrieval of unique genera as *Citricoccus*, *Glycomyces*, *Kocuria*, *Mycolicibacterium*, *Nocardioides*, *Saccharomonospora*, *Spiractinospora* and *Verrucosispora*, which were not recovered in any of the other media. Interestingly, the most oligotrophic media, NPS and SP, allowed the growth of particular taxonomic groups that were not recovered using AIA and SCN media, namely strains affiliated to the genera *Actinomadura*, *Krasilnikoviella*, *Micrococcus* and *Stackebrandtia* (Figure 4B). Nevertheless, strains affiliated to these genera have been cultivated in highly nourishing media—as ISP2, nutrient agar, NZ-amine starch, raffinose–histidine, Sabouraud dextrose and yeast extract agars (Eltamany et al., 2014; Labeda & Kroppenstedt, 2005; Microbispora, 2006; Nishijima et al., 2017; Wang et al., 2009)—attesting their compliance to a broad range of culture compositions. Apart from other methods, the use of innovative culturing media can boost the isolation of new and diverse Actinomycetota. In our study, the source samples (macroalgae) were used to formulate a culture medium that enabled the exclusive isolation of the rare *Krasilnikoviella* genus (Nishijima et al., 2017). The success of this strategy has been previously demonstrated in studies that used sediments and sponge extracts to improve Actinomycetota isolation (Abdelmohsen et al., 2010; Rego et al., 2019).

To better understand the influence of the isolation source—*C. tomentosum* or *C. crispus*—on Actinomycetota diversity, two phylogenetic trees—one comprising all *Streptomyces* strains (Figure 1) and the other all the non-*Streptomyces* isolates (Figure 2)—were constructed with the 376 16S rRNA gene sequences generated in this study (sequences shorter than 900 bp were not considered to the analysis). Results from both trees seem to indicate that the macroalgae species do not have a clear influence on the recovered diversity: closest related strains self-group independently of the macroalgae of origin. This can be exemplified by strains CT-F89 and CC-F41, both identified as *Brachybacterium paraconglomeratum*, with a minimal phylogenetic distance, that were isolated from *C. tomentosum* and *C. crispus*, respectively. These results suggest that the overall ecosystem and culture conditions might play a more significant role in shaping Actinomycetota diversity than the macroalgae species of origin itself. The complex interactions between the bacterial community and the environment—specifically in such a dynamic area as the Atlantic intertidal zone, with exposure to desiccation, temperature and salinity fluctuations, UV radiation and grazing pressure—could strongly influence the composition and abundance of Actinomycetota, highlighting the need to consider the broader ecological context when studying their diversity on living macroalgae.

When analysing the 16S rRNA gene sequences of our collection, potential novel species were identified. According to the 98.65% 16S rRNA gene sequence similarity cut-off to define a new bacterial species (Kim et al., 2014), strains CT-R125, CC-R36, CT-R113 and CC-R104 were selected to further phylogenetic analysis based on their prone taxonomic novelty (Figure 5). CT-R125 and CC-R36, isolated from *C. tomentosum* and *C. crispus*, respectively, represent two potential new species affiliated to the *Streptomyces* genus. The 16S rRNA gene sequence of the first strain has 98.55% similarity with *Streptomyces lusitanus*^{T}, while the second strain presents a similarity of 97.26% with *Streptomyces badius*^{T}. Strains CT-R113 and CC-R104,

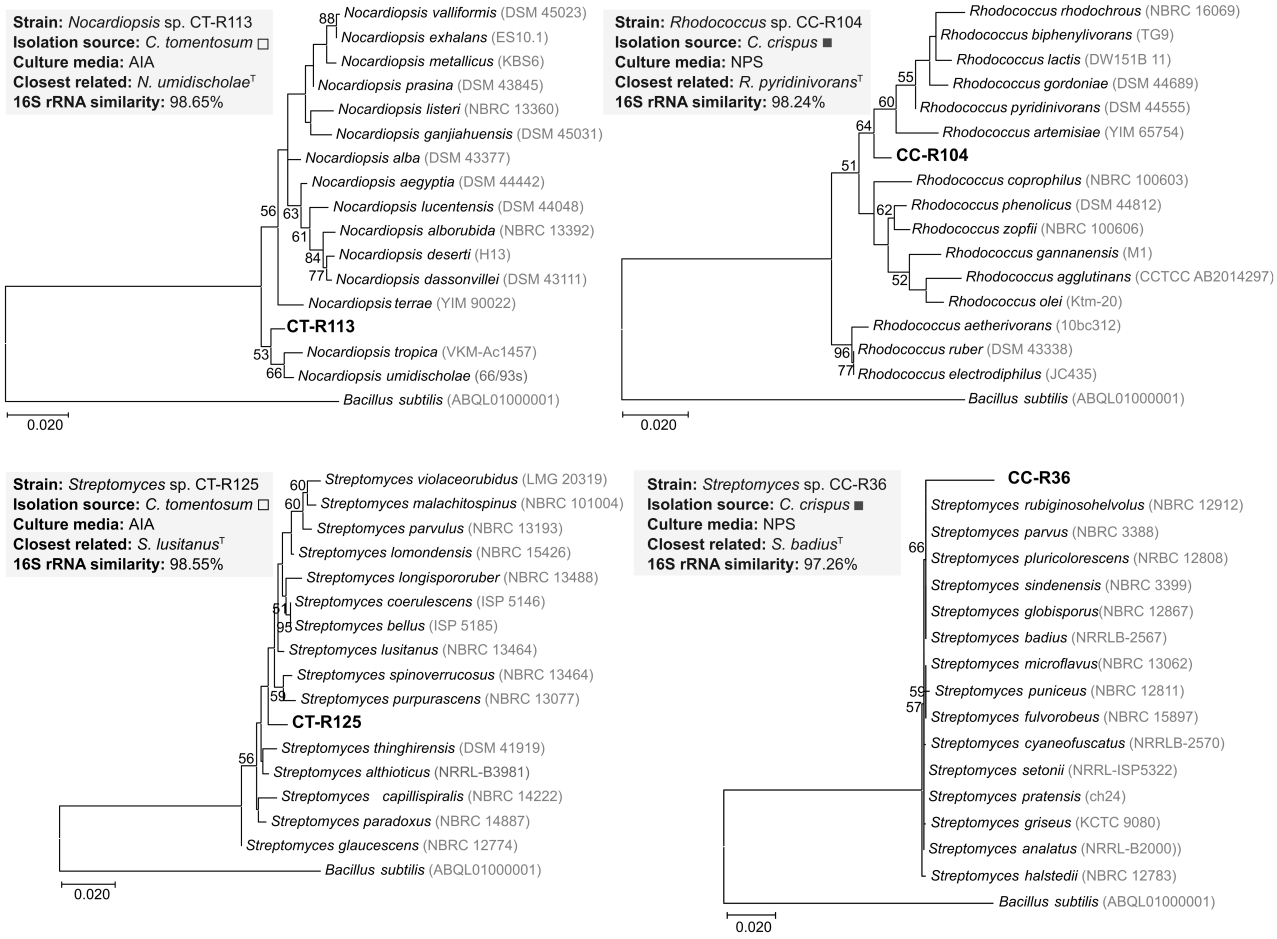

FIGURE 5 Phylogenetic trees for strains CT-R113, CC-R104, CT-R125 and CT-R113, representing potential novel Actinomycetota species recovered from *C. crispus* (■) and C. *tomentosum* (□). The trees were obtained by Maximum Likelihood analysis of the 16S rRNA gene sequences of the 15 closest related type strains associated with each of the potential novel species. The phylogeny test used was the bootstrap method with 1000 replications. *Bacillus subtilis* ABQL01000001 was used as an outgroup. Numbers at nodes represent bootstrap values when higher than 50%. Accession numbers are indicated in brackets.

isolated from *C. tomentosum* and *C. crispus*, represent two potentially new species affiliated with the *Nocardiopsis* and *Rhodococcus* genera, respectively. CT-R113 16S rRNA gene holds a 98.65% similarity with *Nocardiopsis umidischolae*^{T} and CC-R104 has a similarity of 98.24% with *Rhodococcus pyridinivorans*^{T}, making these two strains potential novel species integrating the Nocardiaceae and Nocardiopsaceae families, correspondingly. All the mentioned taxonomic groups are recognized as prolific producers of bioactive secondary metabolites, especially *Streptomyces* (Bennur et al., 2016; Elsayed et al., 2017; Hopwood, 2007), discovering novel bacterial species, and exploration of their metabolomic chemical diversity, a crucial step in compounds discovery, optimization, and design. Additionally, it is important to notice that the resolution of the 16S rRNA gene might not be sufficient for *Streptomyces* species identification, and likely a deeper phylogenetic analysis might reveal a higher number of potential novel species from these macroalgae (Komaki, 2022). The confirmation of the taxonomic novelty of these isolates will require the complete sequencing of their genomes and comparison with the closest type strains. An average nucleotide identity (ANI) analysis of the genomes, rather than solely a comparison based on the 16S rRNA gene, would be necessary, as well as a set of morphological, physiological and biochemical analyses (Kim et al., 2014).

Overall, this culturomics approach yielded 380 actinobacterial strains—highly diverse in metabolic requirements and taxonomy-, constituting the largest collection of macroalgae-associated Actinomycetota reported to date. This collection is dominated by *Streptomyces*, reflecting the adaptability of this species to marine environments and likely significance in host interactions. Potential new taxa were also identified.

Macroalgae-associated Actinomycetota community accessed by metagenomic sequencing

Shotgun metagenomic was used as a culture-independent approach to unravel the Actinomycetota abundance and diversity living in association with *C. crispus* and *C. tomentosum*. A shotgun metagenomic protocol was prioritized instead of an amplicon-based approach, to mitigate potential biases often observed with metabarcoding pipelines. A dataset of almost 140 million reads was generated and classified. Over 50% of prokaryotic relative abundance was attributed to Pseudomonadota taxa, in both macroalgae. Other phyla—Bacteroidota, Cyanobacteria, Planctomycetes, Firmicutes, Verrucomicrobia and Actinomycetota—were also identified. Actinomycetota phylum was particularly abundant in *C. crispus*, ranking as the third most dominant and representing 11.22% of all prokaryotic diversity, while in *C. tomentosum* this value was considerably lower at only 1.73% (Figure 6A, Table S2). Interestingly, this difference was not noticed in the cultivation pipeline, with a higher number of Actinomycetota isolates being retrieved from *C. tomentosum* tissues. In total, 12 Actinomycetota orders, 16 families and 17 genera were identified using shotgun metagenomic (considering only results of relative abundance above the defined threshold of 0.1%, Figure 6B,C, Table S2). When comparing shotgun metagenomic diversity results with the isolates retrieved from the macroalgae, it is possible to see that the overlapping between taxonomic groups is limited: 33% at order level (Micrococcales, Streptomycetales, Propionibacteriales, Pseudonocardiales, Micromonosporares and Streptosporangiales) and 20% at genus level (*Streptomyces*, *Nocardioides*, *Mycolicibacterium*, *Rhodococcus*, *Nocardia and Gordonia*). Previous studies on marine microbial diversity have demonstrated the same pattern where the complementarity of culture-dependent and independent approaches is represented by only a fraction of taxa detected concomitantly (Singh et al., 2012; Sun et al., 2010). A deeper look into the Actinomycetota community revealed by metagenomic sequencing showed that the order Acidimicrobiales (Figure 6B), and the affiliated genus *Ilumatobacter*, (Figure 6C) were the most abundant in both samples. No strain classified within these taxonomic groups was cultured in our work. Other studies on the holobiont of green macroalgae have shown the significant presence of Acidimicrobiia as well (Califano et al., 2020). In contrast to other members of Acidimicrobiales, which are obligate acidophiles, *Ilumatobacter* species grow under neutral or slightly alkaline conditions (Stackebrandt, 2014), being in concordance with the conditions from where the two macroalgae under study were collected. Only three *Ilumatobacter* species—*I. nonamiense*, *I. coccineum* and *I. fluminis*, all retrieved from marine sediments—are reported to date, being this genus described as recalcitrant to cultivation and composed of extremely fastidious strains (Matsumoto et al., 2009; Matsumoto et al., 2013). Previous studies based on 16S rRNA gene sequencing have also shown that these three species are closely related to some uncultured Actinomycetota, including marine sponge symbionts (Montalvo et al., 2005). Tailor-made optimizations on Actinomycetota isolation procedures from macroalgae, including adjustments in media composition, might lead to the discovery of novel and unique species affiliated with *Ilumatobacter* since their presence in the community of both *C. crispus* and *C. tomentosum* is considerably high. The abundance of *Ilumatobacter* in the metagenomic sequencing dataset was more than three-fold higher than the most isolated genus, *Streptomyces*. Apart from *Ilumatobacter*, also the genera *Aquihabitans*, *Actinomarinicola* and *Iamia* presented a higher or similar relative abundance in the

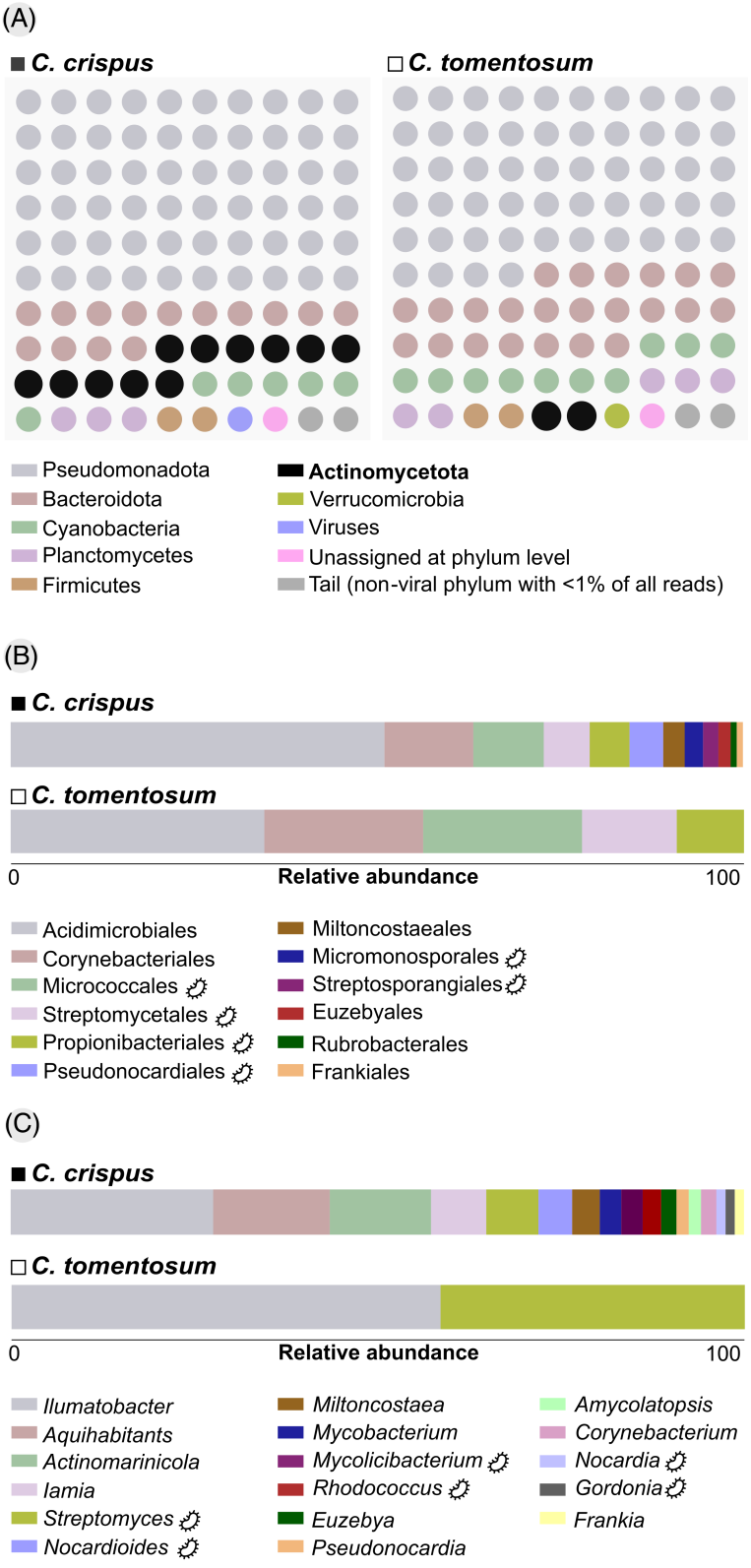

FIGURE 6 Relative distribution of classified reads from *C. crispus* (■) and *C. tomentosum* (□) shotgun metagenomic dataset at (A) phylum-level, with each circle corresponding to 1%, (B) actinobacterial order-level and (C) actinobacterial genus-level. Taxonomic groups retrieved from cultivation are indicated with a bacteria symbol.

C. crispus metagenomic dataset (1.25%, 1.07%, and 0.58%, respectively), when compared with the genus *Streptomyces* (Figure 6C). Although their occurrence in the marine environment (He et al., 2020; Jin et al., 2013; Kurahashi et al., 2009), to our knowledge no species affiliated to these taxonomic groups have been so far isolated from macroalgae. As our findings highlight, prior knowledge about the overall community present in a specific sample can empower the development of tailored cultivation pipelines, enabling the recovery of rare or previously uncultivable Actinomycetota.

Though the analysis of host-specificity of the bacterial community associated with the studied macroalgal species was not the focus of this study, shotgun metagenomic analysis revealed substantial differences in microbial diversity associated with *C. crispus* and *C. tomentosum*. Pseudomonadota is the predominant phylum associated with both macroalgae, comprising over 50% of the prokaryotic relative abundance. However, Actinomycetota was particularly abundant and diverse on *C. crispus*, with the order Acidimicrobiales and the affiliated genus *Ilumatobacter* being the most abundant actinobacterial taxa in both macroalgae. Interestingly, these groups were not isolated through cultivation efforts. While previous studies on macroalgae microbiome have reported that both Gram-negative and Gram-positive communities exhibit host-specificity (Kuba et al., 2021; Li et al., 2022; Sanders-Smith et al., 2020), they have also highlighted that the structure of bacterial communities is more likely associated with functional genes rather than taxonomy (Morrissey et al., 2019; Roth-Schulze et al., 2018). Though we have detected a higher abundance of Actinomycetota in *C. crispus* (Figure 6), the most representative groups within this phylum were the same in both macroalgae. However, a broad comparative study, contemplating more specimens on a higher temporal and biogeographic scale, is necessary to identify the core bacterial community associated with *C. crispus* and *C. tomentosum* and determine the driving factors of such associations.

CONCLUSION

This is the first portrayal of the Actinomycetota community—both culturable and non-culturable—associated with the macroalgae *C. crispus* and *C. tomentosum*. The work here presented not only contributes to enlarging the meagre knowledge available on Actinomycetota communities associated with macroalgae but also provides a collection of 380 unique strains holding great potential for exploration, considering the unmatched biosynthetic machinery encoded by this taxon. Our study highlights the significance of mining untapped marine niches for Actinomycetota discovery. Despite their well-established presence in marine habitats, macroalgae have not been in the spotlight for the isolation and exploration of these bacteria compared to other sources such as sediments, sponges, or corals. Yet, our findings unveil macroalgae as excellent hosts for Actinomycetota, both in number and diversity, paving the way for further studies on their diversity, chemical ecological role and likely biotechnological application. It is important to consider that conclusions drawn from this study are based on a single sampling campaign, providing a snapshot of the microbial community present in the macroalgae at one specific moment. Since microbial communities can vary over time, further investigations must be performed to capture the overall dynamics of the Actinomycetota communities living in macroalgae from the Portuguese coast.

AUTHOR CONTRIBUTIONS

Mariana Girão: Investigation; conceptualization; funding acquisition; writing – original draft; methodology; writing – review and editing; formal analysis; data curation; validation; software; visualization. **Diogo A. M. Alexandrino:** Methodology; data curation; writing – review and editing; software. **Weiwei Cao:** Methodology; writing – review and editing; resources; data curation. **Isabel Costa:** Methodology; writing – review and editing. **Zhongjun Jia:** Methodology; writing – review and editing; resources; data curation. **Maria F. Carvalho:** Conceptualization; funding acquisition; writing – review and editing; validation; formal analysis; project administration; supervision; resources.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

All generated 16S rRNA sequences were deposited in the National Center for Biotechnology Information (NCBI) GenBank database and are available under the accession numbers OR215046-OR215420. Metagenomic sequencing data generated in this study were

deposited in the European Nucleotide Archive (EMBL-EBI) database and are available under the accession numbers ERR12332060 and ERR12332061.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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