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Case Report / Reporte de caso

Hard and soft coral-associated bacteria with pathogenic and probiotic potential

Bacterias asociadas a octocorales y corales escleractinios con potencial patógeno y probiótico

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ABSTRACT

Microorganisms associated with coral reefs play a critical role in coral health and survival. This study aimed to identify difficult-to-culture bacterial isolates associated with the octocorals *Carijoa riisei* and *Leptogorgia alba*, as well as the hermatypic corals *Pocillopora damicornis* and *Pocillopora verrucosa*. Bacterial isolates were identified through 16S rRNA sequencing, resulting in the identification of 18 strains, including several pathogenic bacteria (*Vibrio* sp., *Grimontia indica,* and *Pseudoalteromonas piratica*). Additionally, isolates associated with pathogen-inhibiting properties (R*uegeria profundi, Ruegeria conchae, Pseudoalteromonas luteoviolacea*, and *Pseudoalteromonas gelatinilytica*) were identified. These findings highlight the vulnerability of marine organisms to microbial shifts and provide insight into their responses to environmental stress.

KEY WORDS: Coral reefs, culturable bacteria, microorganisms, octocorals, Eastern Pacific.

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RESUMEN

Los microorganismos asociados a los arrecifes de coral desempeñan un papel fundamental en la salud y supervivencia de los corales. El objetivo de este estudio fue identificar aislados bacterianos de difícil crecimiento, asociados a los octocorales *Carijoa riisei* y *Leptogorgia alba*, y corales hermatípicos *Pocillopora damicornis* y *Pocillopora verrucosa*. Los aislados se identificaron mediante la secuenciación del rRNA16S y se identificaron 18, entre ellas, varias bacterias patógenas (*Vibrio* sp., *Grimontia indica* y *Pseudoalteromonas piratica)*. Además, se identificaron aislados asociados a la inhibición de patógenos (*Ruegeria profundi*, *Ruegeria conchae, Pseudoalteromonas luteoviolacea* y *Pseudoalteromonas gelatinilytica)*. Estos hallazgos muestran la vulnerabilidad de los organismos marinos a los cambios microbianos y proporcionan información sobre sus respuestas al estrés ambiental.

PALABLAS CLAVE: Arrecifes de coral, bacterias cultivables, microorganismos, octocorales, Pacífico Oriental.

Introduction

Coral reefs are among the most biodiverse and productive ecosystems worldwide, supporting approximately 25 % of all marine biodiversity (Spalding et al., 2001; Hughes *et al*., 2010; Carlson *et al*., 2024). The coral holobiont is a complex and dynamic system comprising Dinoflagellates, bacteria, fungi, archaea, endophytic algae, protists, and viruses, microbial representatives from all three domains of life, that establish mutualistic interactions with the host and play a vital role in maintaining coral productivity and homeostasis (Bourne *et al*., 2016; Peixoto *et al*., 2021; Mohamed *et al*., 2023; He *et al*., 2024). Particularly, hermatypic corals and octocorals are integral components of reef ecosystems, forming intricate associations with their symbiotic microorganisms (Rosenberg *et al*., 2007; O'Brien *et al*., 2020; Xiang *et al*., 2022). Therefore, it is evident that specific symbiotic bacteria represent a vital determining group within these corals and octocorals (Lema *et al*., 2012; Hernández-Zulueta *et al*., 2016; Grottoli *et al*., 2018; Hoffmann & Panknin, 2020; Mohamed *et al*., 2023). Several studies have documented the important ecological roles of coral-associated bacteria (Bourne *et al*., 2016; van Oppen & Medina, 2020; Samper *et al*., 2025). For example, some bacteria are involved in the cycling of organic and inorganic matter, resources often limited in reef systems. Diazotrophic bacteria, for instance, have been shown to improve nitrogen fixation (Thompson *et al*., 2015). Symbiotic bacterial communities also help protect their coral hosts from extreme UV radiation during summer (Samper *et al*., 2025) and act as a natural defense barrier by synthesising antimicrobial compounds (McDevitt-Irwin *et al*.,

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2017). Indeed, bacterial groups with antimicrobial activity exhibit increased flexibility and dynamic responses under disease stress, enabling rapid protection against invading pathogens (Bourne *et al*., 2016; van Oppen & Medina, 2020; He *et al*., 2024).

The composition of bacterial assemblages is shaped by multiple factors that regulate microbial community structure, including the host-specificity of certain bacterial groups (van de Water *et al*., 2018; Freire *et al*., 2019). The remaining components of the bacterial community are influenced by local environmental conditions (temperature, pH, oxygen concentration, and nutrients) (Bourne *et al*., 2016; van Oppen & Medina, 2020) as well as by competitive interactions between bacterial communities (Zhang *et al*., 2020; Cheng *et al*., 2023). Characterizing the bacterial communities associated with marine invertebrates provides valuable insight into the ecological roles of these microorganisms (Rappé & Giovannoni, 2003; Falkowski *et al*., 2008; Ameen *et al*., 2021). However, studying bacteria in laboratory settings remains highly challenging, as only 0.01-0.1% of marine bacterial cells form colonies using standard plating techniques (Kogure *et al*., 1979; Caycedo Lozano *et al*., 2021). These low recovery rates are primarily due to technical limitations. Nevertheless, the study of culturable bacteria enables the exploration of microbial diversity, supports fundamental biological research, and facilitates the discovery of novel bioactive compounds with potential biotechnological applications (Kogure *et al*., 1979; Overmann *et al*., 2017).

Culture media supply essential nutrients for bacterial growth, including carbon sources, nitrogen, and mineral salts (Bonnet *et al*., 2019; Caycedo Lozano *et al*., 2021). However, bacteria in their natural habitats access a broader array of environmental resources. A major obstacle to isolating many prokaryotic taxa is the disruption of symbiotic or cooperative relationships between microorganisms that depend on shared metabolic growth factors, chelating agents, or signaling molecules (Lewis *et al*., 2021; Zhang *et al*., 2024). Therefore, isolating bacterial strains is essential to gain deeper insights into the microbial communities associated with corals and octocorals from the Mexican Central Pacific (MCP). In this study, we focused on bacterial isolates that cannot be cryopreserved due to their inability to regrow in culture media.

Material and Methods

Obtaining bacterial isolates

Bacteria were isolated from the octocorals *Carijoa riisei* and *Leptogorgia alba* and the stony corals *Pocillopora damicornis* and *P. verrucosa*. Samples were collected by technical diving at several sites in the MCP (Table 1), during the presence of the El Niño phenomenon, September-November 2023. The average water temperature during sampling was 30.8 °C, and the collection depth ranged from 6 to 10 meters. For the octocorals, branch fragments approximately 2-3 cm in length were collected from three different colonies of each species. Samples were stored on ice at 4 °C and transported to the Laboratorio de Ecología, Conservación y Taxonomía (LEMITAX) at the Universidad de Guadalajara, Mexico. Coral fragments were homogenized using a mortar and pestle and then placed in sterile seawater tubes. The samples were then subjected to constant agitation

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at one-minute intervals. The culturing strategy was designed to recover marine heterotrophic bacteria. Samples were plated onto Zobell marine agar (1 g of yeast, 5 g of bactopeptone, 1 mL of 1% ferric chloride, 13 g of agar in 1 L of seawater filtered through 0.22-micron pore-size filters) and incubated (LSIS-B2V/ICV55-INCUCELL V) for two to five days at 28 °C. Conversely, fragments approximately 3-5 cm long were collected from three individuals' colonies of each species of hermatypic coral. Field processing followed the methodology of Lampert *et al*. (2016), with some modifications. Mucus samples were collected using sterile swabs and inoculated directly onto Zobell marine agar plates. This process was carried out in a sterile area delimited by burners. These Petri dishes were kept at room temperature for 48 hours and then refrigerated for transport to the laboratory.

During bacterial purification, only isolates that grew in the initial subculture but failed to grow in subsequent subcultures were selected. According to the methodology proposed by Gerhardt *et al*. (1981), isolates exhibiting distinct morphological traits (shape, margin, texture, pigmentation, and appearance) were selected. Depending on the case, subcultures were performed using one of two methods: toothpick inoculation or direct colony streaking. In the first instance, the bacterial cells were inoculated into test tubes containing 2 ml of Zobell marine broth [yeast (1 g), bactopeptone (5 g), and 1% ferric chloride (1 mL), dissolved in 1 L of seawater filtered through 0.22-micron pore-size filters]. Subsequently, the cultures were incubated under constant agitation. For the second strategy, a colony was selected with a sterile loop for direct streaking and inoculated onto Petri dishes containing marine Zobell medium. In both approaches, cultures were incubated for 48 hours at 28 °C or until bacterial growth was observed.

Molecular Identification

Bacterial isolates were identified by extracting genomic DNA using the "DNeasy Blood and Tissue Kit" (QIAGEN®), following the manufacturer's instructions. The 16S rRNA gene was amplified using polymerase chain reaction (PCR) with a primer pair at a concentration of 10 mM: 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC CTT GTT ACG ACT T-3') (Frank *et al*., 2008). All PCR reactions were performed using 20 µg/mL of DNA in a total volume of 25 µL with the DreamTaq Green Master Mix (2X) Kit (#K1081, Thermo Scientific®) under the following conditions: 1) Initial denaturation at 94 °C for 4 min; 2) 35 cycles at 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 45 s; 3) Final extension at 72 °C for 10 min. Distilled water was used as a negative control, verifying the presence of the 1500 bp amplicon, corresponding to regions V1-V9 of the 16S rRNA gene, by 1 % agarose gels visualized with SYBR® Safe staining. The PCR fragments were purified using the "GenEluteTM Gel Extraction" Kit (NA1111, Sigma-Aldrich®), following the manufacturer's instructions. Sequencing was performed using the TaqBigDye Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, Foster City, USA), purified with ethanol, and visualized in SeqStudio Genetic Analyzer at the Laboratorio Nacional de Identificación y Caracterización Vegetal (LaniVeg), Centro Universitario de Ciencias Biológicas y Agropecuarias, at the Universidad de Guadalajara. Sequences of ~1400 bp were obtained, and the quality was assessed using Chromas® software (Technelysium, DNA Sequencing Software). Sequence alignment and identification were performed using BLAST against the Nucleotide

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database of GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/). A sequence similarity of over 98 % was deemed sufficient for species-level identification, while a 90% similarity was used for genus-level assignment.

Results and Discussion

A total of 18 bacterial isolates failed to grow in subsequent subcultures. Of these, two were from *C. riisei*, six from *L. alba*, eight from *P. damicornis*, and two from *P. verrucosa*. These isolates initially demonstrated growth on general solid culture medium (Figure 1A). Following the isolation process, only a small number of colonies were observed (Figure 1B). Despite attempts to enhance cell growth through liquid cultures, reseeding, or increasing the incubation temperature to 35 °C and extending the incubation period to 72 hours, no further growth was observed. Additional enriched media were therefore tested, including Trypticase Soy Agar (TSA), Luria-Bertani (LB), and selective media such as MacConkey agar and Thiosulfate Citrate Bile Sucrose (TCBS) agar. However, none of these media were found to support bacterial growth. These results form part of a broader study investigating bacterial isolates associated with the coral and octocoral species evaluated in this work. These strains encompass ~600 bacterial isolates, and their characterization is currently underway.

Only the isolates that failed to demonstrate bacterial growth in subcultures were identified at the molecular level to preserve any remaining viable cells (Table 1). The bacteria *Ruegeria profundi* and the unidentified isolate CrS1SC.3 were found to be associated with *C. riisei*. The genera *Grimontia*, *Pseudoalteromonas*, *Shewanella*, *Ruegeria* and *Vibrio* were identified as being associated with *L. alba*. Among the isolates from scleractinian corals, those associated with *P. damicornis* belonged to the genera *Alteromonas*, *Fictibacillus*, and *Pseudoalteromonas*. Meanwhile, *Chromoalobacter israelensis* and *Shewanella seohaensis* were isolated from *P. verrucosa*.

This study reports bacterial isolates associated with octocorals and hermatypic corals that could not be successfully reseeded and cryopreserved, and are referred to as ‘difficult-to-culture’ bacteria (Vartoukian *et al*., 2016). Bacterial cultivation requires specific conditions to be met to ensure their viability and growth (nutrients, water, carbon, nitrogen sources, and mineral salts) (Caycedo Lozano *et al*., 2021). However, as reported by Cocolin (2010), enrichment and growth techniques in microbiological media can markedly alter the original microbiota composition of a sample. This is because certain microbial species may outcompete others, leading to the underrepresentation or complete loss of uncommon or stressed populations. Furthermore, environmental factors such as sea temperature fluctuations, nutrient availability, competitive interactions, and host specificity are key variables that determine the presence of specific bacterial groups (Zhang *et al*., 2020). These variables can also contribute to the development of non-culturable bacterial cells, as laboratory conditions rarely replicate the exact environmental niches required for growth (Zhang *et al*., 2024). Bacteria can enter a dormant or ‘viable but not culturable’

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state to endure environmental stressors, rendering them undetectable by conventional culture methods (Shleeva *et al*., 2011; Vartoukian *et al*., 2016). This limitation of traditional cultivation approaches hampers the accurate assessment of microbial biodiversity in natural ecosystems. Therefore, the findings presented here should be complemented by the characterization of the remaining culturable bacteria associated with the sampled coral species. In addition, integrating amplicon-based metabarcoding analyses would provide a more comprehensive overview of the associated microbial assemblages.

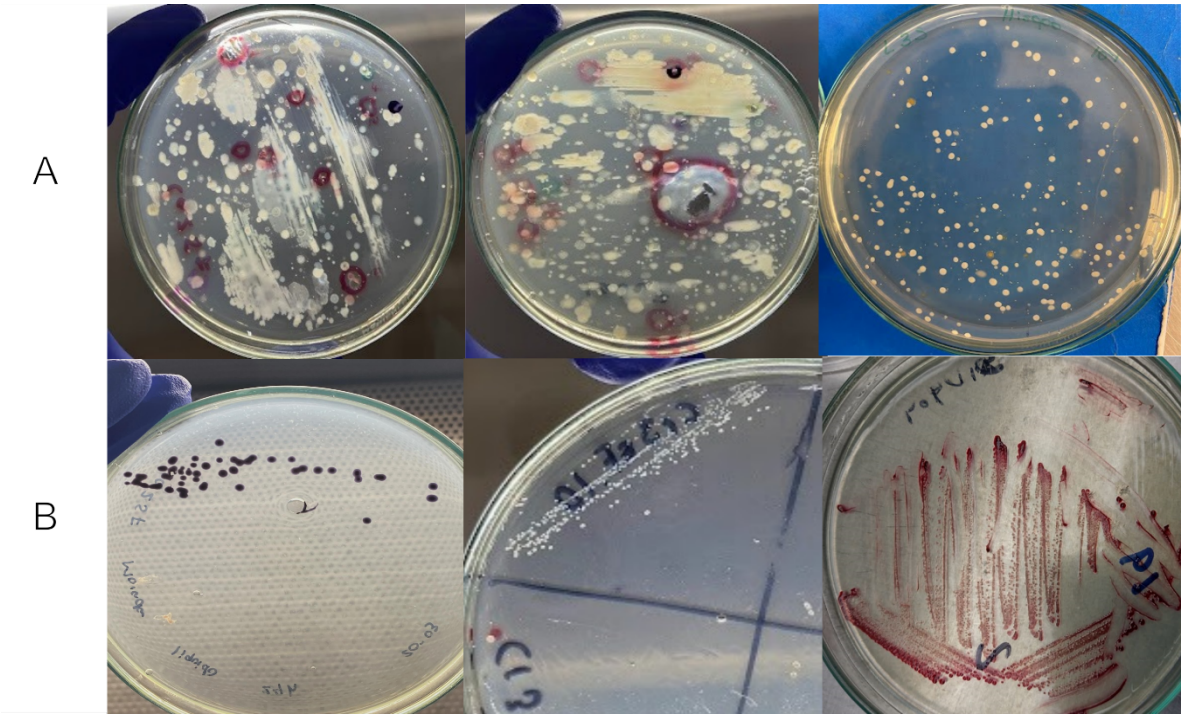

Figure 1. Coral-associated bacterial isolates.

A) Bacterial isolates from the initial concentrates sample. B) Subcultures with little growth of morphologically different colonies.

Source: own.

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Table 1. Bacterial isolates present in *C. riisei, L. alba, P. damicornis* and *P. verrucosa.*

Isolation code	Organism from which isolated	Collection site	Bacterial species	NCBI-GenBank Access Number SUB14676057
CrS1SC.3	*C. riisei*	Manzanillo, Colima	S/I	-
CrJ1SC.1	*C. riisei*	Manzanillo, Colima	[2]*Ruegeria profundi*	PQ222729
LS1SC.2	*L. alba*	Manzanillo, Colima	[4]*Shewanella submarina*	PQ222723
LJ2SC.3	*L. alba*	Manzanillo, Colima	[3]*Vibrio* sp.	PQ222724
LS2SC.4	*L. alba*	Manzanillo, Colima	[3]*Grimontia indica*	PQ222725
LJ1SC.1	*L. alba*	Manzanillo, Colima	[3]*Pseudoalteromonas piratica*	PQ222726
LJ1SC.3	*L. alba*	Manzanillo, Colima	[2]*Ruegeria conchae*	PQ222727
LJ1SC.5	*L. alba*	Manzanillo, Colima	[2]*Ruegeria conchae*	PQ222728
S1D4.9	*P. damicornis*	Bahía Chamela, Jalisco	[1]*Pseudoalteromonas rubra*	PQ222731
S2D2.5	*P. damicornis*	Bahía Chamela, Jalisco	[2]*Pseudoalteromonas luteoviolacea*	PQ222732
S8D1.7	*P. damicornis*	Bahía Chamela, Jalisco	[2]*Pseudoalteromonas gelatinilytica*	PQ222733
S8D1.8	*P. damicornis*	Bahía Chamela, Jalisco	[1]*Alteromonas abrolhosensis*	PQ222734
S6D2.9	*P. damicornis*	Cuastecomatitos, Jalisco	[3]*Pseudoalteromonas piratica*	PQ222736
S6D2.6	*P. damicornis*	Cuastecomatitos, Jalisco	[1]*Pseudoalteromonas phenolica*	PQ222737
S1D1B.7	*P. damicornis* *	Cuastecomatitos, Jalisco	S/I	-
S8D3B.8	*P. damicornis* *	Carrizales, Colima	[4]*Fictibacillus solisalsi*	PQ222735
S1V6.4	*P. verrucosa*	Carrizales, Colima	[1]*Chromohalobacter israelensis*	PQ222730
S8V2B.2	*P. verrucosa* *	Cuastecomatitos, Jalisco	[4]*Shewanella seohaensis*	PQ222738

*Correspond to corals with bleaching during sampling. Superscripts indicate the activity reported: 1) Antimicrobial ability. 2) Antimicrobial ability against pathogens associated with coral diseases. 3) Pathogens related to coral diseases. 4) There are no reports related to pathogenicity or antimicrobial activity. S/I: Unidentified

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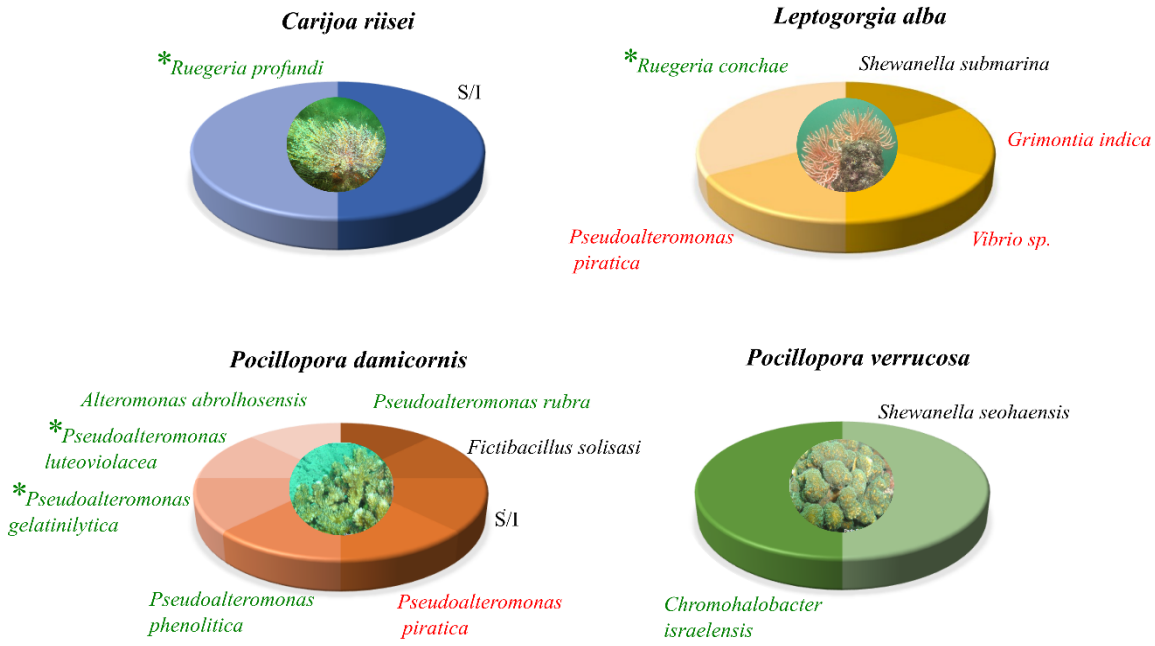

Figure 2. Bacterial isolates associated with *Carijoa riisei, Leptogorgia alba, Pocillopora damicornis* and *P. verrucose.*

Names in green color indicate species related to antimicrobial capacities. * Species related to defense against coral disease pathogens. Red names indicate pathogenic species related to coral diseases. Names in black are from no reports related to pathogenicity or antimicrobial activity.

The 2023 El Niño-Southern Oscillation (ENSO) event was considered an exceptional phenomenon, characterized by unusual atmospheric disturbances and significant increases in sea surface temperatures (Pérez- de Silva *et al*., 2022; Peng *et al*., 2024). These thermal shifts have been identified as an important contributing factor to coral bleaching (Reimer *et al*., 2024) and have been reported to alter the abundance and composition of coral-associated microorganisms, particularly pathogenic bacteria (Gibbin *et al*., 2019). Regarding the bacterial isolates reported in this study, it seems probable that this event induced stress in the corals and their associated bacterial assemblages, promoting specific interactions that inhibited bacterial proliferation and prevented many taxa from growing on standard laboratory culture media.

Vibrio and *Pseudoalteromonas* represent one of the most prevalent bacterial groups associated with coral diseases, as evidenced by numerous studies (Tout *et al*., 2015; Gibbin *et al*., 2019). It is well documented that species such as *Vibrio coralliilyticus*, *Vibrio shilonii*, and *Pseudoalteromonas piratica* are pathogens implicated in tissue loss in corals and the onset of diseases such as white syndrome and yellow band disease (Beurmann *et al*., 2017; Ben-

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Haim *et al*., 2003a,b; Jayasreea *et al*., 2021). A correlation has been observed between the presence of *V. coralliilyticus* and elevated sea surface temperatures in colonies of *P. damicornis*. Simultaneously, in the Atlantic and Mediterranean seas, there have been reports of bacterial diseases affecting octocorals, where the main cause has been *V. coralliilyticus* (Weil *et al*., 2017). Additionally, *Grimontia indica*, isolated from *Leptogorgia alba*, harbors virulence-related genes such as ompU, which are associated with pathogenicity in *Vibrio* strains, suggesting its potential as an opportunistic pathogen (Singh *et al*., 2014). However, the present study identified the presence of these bacterial groups in the octocoral *L. alba* and the hermatypic coral *P. damicornis*. Notwithstanding the presence of these genera, no visible disease-related lesions were observed on the coral hosts.

Additionally, species exhibiting antimicrobial and algaecidal properties, including *Alteromonas abrolhosensis*, *Pseudoalteromonas phenolica*, *P. rubra*, and *Chromohalobacter israelensis*, have been identified in association with stony corals of the genus *Pocillopora* (Isnansetyo *et al*., 2003; John *et al*., 2020; Wang *et al*., 2021; Jia *et al*., 2023). Some of these species have been shown to directly inhibit the pathogens responsible for coral diseases. The presence of these bacteria associated with *Pocillopora damicornis and P. verrucosa* suggest that these corals harbor a bacterial assemblage that may function as a protective barrier against infectious agents. For instance, *Ruegeria conchae* and *R. profundi* have demonstrated inhibitory effects against *Vibrio* pathogens (Miura *et al*., 2019). Specifically, *R. profundi* has exhibited probiotic properties by suppressing the proliferation of *Vibrio coralliilyticus*, thereby reducing its pathogenicity, promoting microbiome homeostasis, and enhancing the holobiont's resilience to pathogen-induced stress (Xu *et al*., 2024). Furthermore, the presence of the *Ruegeria* group in association with *C. riisei* and *L. alba* may play a pivotal role in pathogen defense, microbiome regulation, and the sustainability of the studied octocoral species.

Pseudoalteromonas luteoviolacea and *P. gelatinilytica*, which were isolated from *P. damicornis*, have been reported to exhibit antimicrobial activity against *Vibrio* pathogens, including *V. coralliilyticus* and *V. alginolyticus* (Vidal-Dupiol *et al*., 2011; Gibbin *et al*., 2019; Fazeli *et al*., 2021; Jayasreea *et al*., 2021). The isolation of these bacteria from visually healthy coral colonies suggests they may play an important role in the protection of the host against pathogens such as *P. piratica*, which is also associated with *P. damicornis*. In contrast, bacteria such as *Fictibacillus solisalsi* and *Shewanella seohaensis*, identified in bleached coral colonies, have no known associations with bleaching, pathogenicity, or probiotic activity. This highlights the need to refine bacterial isolation methodologies to gain a deeper understanding of the ecological functions of key bacterial species in disease dynamics and their impact on coral health.

Conclusions

The isolation of culturable bacteria is imperative for comprehending the vital roles of microorganisms in the environment, for identifying bacterial species that serve as bioindicators of diseases or contribute to the regulation of microbiomes in marine organisms and substrates. In this study, we observed the presence of known pathogens, such as *Pseudoalteromonas piratica*

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and *Vibrio* species, associated with the octocoral *L. alba*. Furthermore, we confirmed the presence of pathogens in *P. damicornis* in response to rising sea temperatures. Moreover, the presence of probiotic bacterial species with antimicrobial properties against the aforementioned pathogens was recorded in both octocorals and scleractinian corals, including *Ruegeria conchae*, *R. profundi*, *Pseudoalteromonas luteoviolacea*, and *P. gelatinilytica*. It is imperative to acknowledge the significance of identifying non-culturable bacteria, as this facilitates the consideration of potential requirements that should be taken into account for the future characterisation of bacterial communities. This study lends further support to the notion that rising sea temperatures are adversely affecting marine organisms. It is recommended that future studies consider utilising an integrated approach of 16S rRNA ribosomal gene amplicon analysis (metabarcoding) and traditional microbiological methods, such as isolation and culture. It´s essential to study the diversity of microorganisms, including non-culturable microorganisms, for developing a more comprehensive understanding of the plasticity of bacterial assemblages in response to environmental change, as well as for evaluating the true potential of these microorganisms as regulatory agents that may support host survival under environmental stress.

Authors' contribution

Conceptualization of work: E.A.C. methodology development: E.A.C., M.E.L.C; experimental validation: E.A.C., M.E.L.C; analysis of results: E.A.C., J.H.Z; data management: E.A.C; manuscript writing and preparation: E.A.C., J.H.Z; writing, revising and editing: M.E.L.C., F.A.R.Z., C.M.G.V., A.L.P., E.G.D., A.O.O. Fund acquisition: J.H.Z., F.A.R.Z., C.M.G.V., A.L.P.

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Declaration of informed consent

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Conflict of interest

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"The authors declare that they have no conflicts of interest".

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