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Effect of Various Local Anthropogenic Impacts on the Diversity of Coral Mucus-Associated Bacterial Communities

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Abstract: The global continued decline in coral reefs is intensifying the need to understand the response of corals to local environmental stressors. Coral-associated bacterial communities have been suggested to have a swift response to environmental pollutants. This study aims to determine the variation in the bacterial communities associated with the mucus of two coral species, *Pocillopora damicornis* (Linnaeus, 1758) and *Stylophora pistillata* (Esper, 1792), and the coral-surrounding seawater from three areas exposed to contamination at the Jordanian coast of the Gulf of Aqaba (Red Sea), and also explores the antibacterial activity of these bacteria. Corals were collected from three contaminated zones along the coast, and the bacteria were quantified and identified by conventional morphological and biochemical tests, as well as 16S rRNA gene sequencing. The average number of bacteria significantly varied among the coral mucus from the sampling zones and between the coral mucus and the surrounding seawater. The *P. damicornis* mucus-associated bacterial community was dominated by members of the classes Gammaproteobacteria, Cytophagia, and Actinomycetia, while the mucus of *S. pistillata* represented higher bacterial diversity, with the dominance of the bacterial classes Gammaproteobacteria, Actinomycetia, Alphaproteobacteria, and Bacilli. The effects of local anthropogenic impacts on coral mucus bacterial communities were represented in the increased abundance of bacterial species related to coral diseases. Furthermore, the results demonstrated the existence of bacterial isolates with antibacterial activity that possibly acted as a first line of defense to protect and maintain the coral host against pathogens. Indeed, the dynamics of coral-associated microbial communities highlight the importance of holistic studies that focus on microbial interactions across the coral reef ecosystem.

Keywords: bacterial communities; coral mucus; Gulf of Aqaba; seawater; *Pocillopora damicornis* and *Stylophora pistillata*

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1. Introduction

Marine ecosystems are among the largest and most diverse ecosystems, and are characterized by their biological productivity [1]. Coral reefs host about 25% of marine species, even though they represent less than 0.1% of the marine environments [2]. However, this fundamental ecosystem is degenerating over time, since 27% of coral reefs were destroyed in the last few decades worldwide, and the rest are under threat of being lost [2,3].

Coral hosts dense, dynamic, and highly diverse consortia of microorganisms, such as *dinoflagellates*, *Bacteria*, *Archaea*, viruses, and fungi, forming a complex mutualistic relationship with corals (coral holobiont) [4]. These microorganisms inhabit the range of ecological niches provided by corals, such as the surface mucus layer, tissue layers, and the skeleton [5]. The interactions of corals and their associated microbial community contribute to various aspects of coral biology, including nutrition, protection, growth, survival, and their general health status [6,7]. However, the mechanisms for acquiring bacterial associations with host corals are poorly understood.

Different processes of the coral holobiont are promoted by microbial communities associated with corals, including the carbon cycle [8], the sulfur cycle [9], phosphorus fixation, metal homeostasis, organic matter treatment [10], antibiotic production [11], and secondary metabolism [10]. Many coral-associated bacteria protect corals, for example (coral *Acropora palmata* (Lamarck, 1816) [11]), by secreting antimicrobial compounds to prevent the entry of pathogens or other exogenous bacteria. Additionally, some coral-resident bacteria actively predate upon some pathogens within coral mucus [12]. Indeed, the disruption or destabilization of the coral holobiont can affect the host's fitness and ecosystem dynamics [12].

The diversity of coral-associated bacterial communities is affected by local environmental factors. For example, a change in the bacterial communities associated with the coral *Acropora hemprichii* (Ehrenberg, 1834) was induced by enrichment with inorganic nutrients in a coral reef habitat [13] and caused outbreaks of coral disease [14,15]. Furthermore, increased nutrients coupled with overfishing have indirectly affected coral bacterial communities by promoting algal growth that induces coral mortality by microbes [16]. Discharged sewage enters coastal ecosystems, carrying high loads of inorganic nutrients, sediments, and organic compounds, which can have deleterious effects on coral reefs [17,18]. Moreover, sewage is also expected to introduce many new microbial taxa belonging to sewage-associated human pathogens and may consequently induce the development of coral disease [19,20]. The acidification of marine water is a consequence of anthropogenic CO_2 emissions that is negatively impacting coral reefs. The study conducted by Shore et al. [21] on three coral species sampled from three sites with different seawater pH levels revealed that acidification has multiple consequences on coral bacterial communities, and suggests that the abundance of bacterial species *Endozoicomonas* may be an indicator of the coral's response to the acidification of marine environments. Generally, coral-associated bacterial communities' responses to environmental stressors are consistent across multiple stressors, with increased relative abundances for members of the Vibrionales, Flavobacteriales, Rhodobacteriales, and Alteromonadales [22].

The mechanisms of coral interaction with the epi-biotic marine bacteria are known to play a significant role in the marine ecosystem. One of the potential mechanisms involves the maintenance of antimicrobial chemicals against pathogens [23]. It has been hypothesized that the coral holobiont may protect corals from pathogens by occupying niches and/or producing antibiotics [4]. The mucus of several coral species is characterized by their secretion of allochemicals with antimicrobial properties [24–26]. A previous study reported that a high percentage (30%) of bacteria isolated from coral species have antimicrobial capabilities [27]. Nithyanand and Pandian [28], for example, reported the presence of actinomycetes in the coral mucus of *Acropora digitifera* (Dana, 1846), which had high activity against pathogens.

The Gulf of Aqaba is one of the unique aquatic ecosystems that has one of the world's richest coral communities [29]. However, the Gulf of Aqaba is highly affected by anthropogenic stressors in the Middle East [30]. Coral reefs on the Jordanian coast of the Gulf of Aqaba have been subjected to various sources of environmental stressors in the last few years [29]. The main stressors include coral reef damage caused by tourism, oil spills, air pollution associated with land transportation, disposal of solid waste, phosphate dust deposition from ship loading activities, chemical and thermal pollution from coastal mega industries, and sewage discharges into the marine environment [31,32].

The aim of the current study was to assess the bacterial communities associated with the mucus of two species of coral and the coral-surrounding seawater from sites affected by various types of anthropogenic stressors in the Gulf of Aqaba (Jordan), to explore their antimicrobial activities, and to explore whether the diversity of and spatial variations in bacterial communities are affected by the sources of anthropogenic stressors in the study areas.

2. Materials and Methods

2.1. Sample Collection

Samples from two hard coral species (*S. pistillata* and *P. damicornis*) were collected by SCUBA divers (at a depth of 4–11 m) from three sites on the Jordanian coast of the Gulf of Aqaba: the Industrial Zone (I.Z), the Public Beach (P. Beach), and the Phosphate Berth (P. Berth) (Figure 1). The selected sites vary in terms of the source and level of anthropogenic stressors (Table S1). Coral samples were collected during the spring season (March 2016). Three replicates of both coral species were collected from each site. The samples were immediately placed in plastic tubes underwater and transported to the laboratory in an icebox within one hour of collection, as described by Koren and Rosenberg [33]. The seawater surrounding the coral species was also sampled using pre-sterilized 50 mL falcon tubes. The samples were stored at 4 °C and transported immediately to the laboratory.

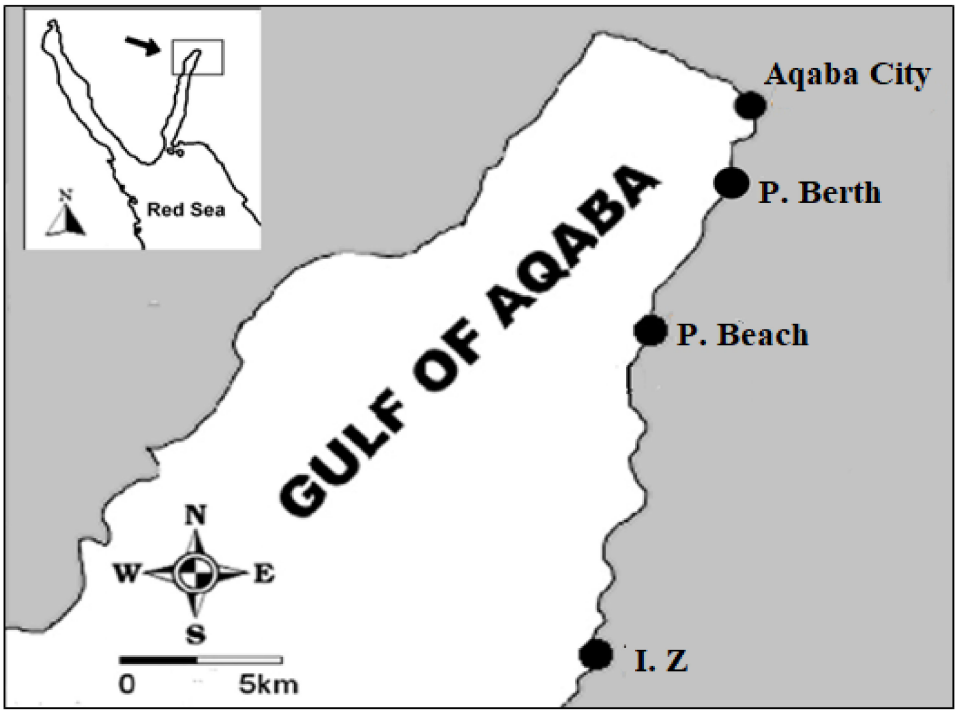

Figure 1. Sample sites in the Gulf of Aqaba (Jordan) (I.Z: Industrial Zone, P. Beach: Public Beach, and P. Berth: Phosphate Berth).

2.2. Mucus Extraction

The extraction of coral mucus was carried out as described by Omry and Eugene [34]. Briefly, the coral samples were broken into small pieces and placed in sterile centrifuge tubes. The coral samples were then centrifuged for 10 min at 10,000 rpm at 4 °C to remove the mucus. After centrifugation, the mucus was collected in a 2 mL Eppendorf tube and stored at 4 °C until processing. Extraction was conducted in triplicate.

2.3. Isolation and Enumeration of Bacteria

Tenfold serial dilution of coral mucus and seawater samples was prepared and cultivated on marine agar (MA) media (Marine Agar 2216, PanReac, Castellar del Vallès, Spain). MA plates were incubated at 30 °C for 48 h. The viable plate count method was used to enumerate the viable bacteria of the samples and expressed in colony-forming units per ml (CFU/mL) as described by Lampert et al. [35]. The grown colonies were sub-cultured on new fresh MA plates several times to obtain a pure culture of bacterial isolates.

2.4. Morphological and Biochemical Characterization

The bacterial isolates were identified by performing a series of morphological and biochemical tests according to Bergey's manual of determinative bacteriology [36] (Tables S2 and S3). The characterization of the bacterial isolates was conducted by the Gram reaction; after that, the colonial morphology was determined. A series of selective media (MacConkey agar, Pseudomonas agar, Simmons Citrate agar, Eosin Methylene Blue agar, and Salmonella agar) was used to characterize these isolates, as described by Garrity et al. [37]. All biochemical tests were performed according to the standard protocols using filtered seawater for media preparation to fulfill the halophilic requirement of marine bacteria.

2.5. Molecular Identification

Bacterial isolates that were not identified using conventional biochemical tests were subjected to identification based on molecular techniques (16S rRNA gene sequencing). Genomic DNA from the coral mucus-associated bacterial isolates was extracted using Bacteria DNA Extraction Kits (Thermo Fisher Scientific Inc., Waltham, MA, USA) following the manufacturer's instructions. The extracted DNA was quantified using an UV-spectrophotometer (DNA concentration > 50 ng/µL).

The 16S rRNA gene of the bacteria was amplified from the extracted genomic DNA using the following eubacterial universal primers: forward primer (5′ AGAGTTTGATCCTGGCTCAG 3′) and reverse primer (5′ GGTTACCTTGTTACGACTT 3′) [35]. PCR was performed in a 25 mL reaction mixture with initial denaturation for 3 min at 95 °C, 40 cycles consisting of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min, and a final extension step of 5 min at 72 °C. The amplification of the 16S rRNA gene was confirmed by running the amplification product in 1% agarose gel.

Sequencing of the amplified PCR products was performed at Macrogen Inc., Seoul, Korea. The obtained sequences were matched with previously published sequences available in NCBI using BLAST (http://blast.ncbi.nlm.nih.gov/Blast (accessed on 8 April 2018)) [38] and the BLASTn tool. Molecular phylogenetic analysis was conducted using the neighbor-joining statistical method [39]. The trees were drawn to scale, with the branch lengths measured in the number of substitutions per site. Phylogenetic analyses were conducted in MEGA11 (version 11.0.11) [Institute of Molecular Evolutionary Genetics, State College, PA, USA]. To validate the reproducibility of the branching pattern, a bootstrap analysis was performed with 500 replications [40].

2.6. Antimicrobial Activity Assay

Screening of the isolates' antibacterial activity was conducted following the agar well diffusion method [41]. The reference bacterial strains were freshly prepared in nutrient broth and incubated at 37 °C for 18 h. The turbidity of each culture was adjusted to 0.5 McFarland standard, and each culture was spread onto nutrient agar media plates. Equidistant wells (8 mm diameter) were created in the inoculated nutrient agar plates using a sterile cork borer. The pure cultures' bacterial isolates containing the antimicrobial were inoculated in marine broth for 48 or 72 h (according to the growth rate of the isolate). An amount of 100 µL of the pure culture broth was added to each well and the plates were incubated at 37 °C for 24 h. The antibacterial activity was measured in terms of the diameter of the inhibition zone in triplicate. The positive control for these experiments was ampicillin (10 mg mL^{-1}), whereas the negative control was marine broth media. The

antibacterial activity of the extract was evaluated against four test bacteria: *Pseudomonas aeruginosa* ATCC13048 and *Serratia marcescens* ATCC27117 (as representatives of Gram-negative bacteria), as well as *Micrococcus luteus* ATCC9341 and *Bacillus cereus* ATCC11778 (as representatives of Gram-positive bacteria).

2.7. Statistical Analyses

Statistically significant differences in the CFU/mL values with different factors (coral species, sampling sites, and sample matrix (coral mucus vs. seawater)) were determined by a one-way ANOVA test using PAST software [42]. Differences were considered significant at $p < 0.05$. The data met the assumptions for independence and normality. Dunn's post hoc test was applied to the obtained significant calculated statistical differences [42]. To identify the shared and unique bacterial isolates from the coral mucus and surrounding seawater bacterial communities, a Venn diagram was constructed using Venny 2.0 [43]. Similarity percentage analysis (SIMPER) was used to represent the species of bacterial communities contributing to the differences between sampling sites and between the bacterial matrices (mucus vs. seawater), and analyses were performed in PRIMER v. 6 [44]. The visualization of the spatial variation in coral mucus and the surrounding seawater bacterial communities was conducted using unconstrained ordination plots with principal coordinate analysis (PCoA) based on the Euclidean similarity index created by PAST software (version 4.10) [42].

3. Results

3.1. Coral Mucus and Seawater Viable Bacterial Count

The viable bacterial count was determined for the coral mucus of both *S. pistillata* and *P. damicornis* (Figure 2). The highest viable bacterial number was detected in the mucus samples of *P. damicornis* obtained from the Phosphate Berth, whereas the lowest viable bacterial count was detected in the mucus samples of both corals obtained from the Industrial Zone. Significant statistical differences (one-way ANOVA, $p < 0.05$) were detected between the bacterial counts (in CFU/mL) from the three sampling sites, where F (2,6) = 5779 and $p = 1.4 \times 10^{-10}$ in case of *P. damicornis*. Dunn's multiple comparison test found that the mean value of the bacterial counts was significantly different between the Industrial Zone and Phosphate Berth sites ($p = 0.00729$). However, in the case of *S. pistillata*, F (2,6) = 2640 and $p = 1.46 \times 10^{-9}$, and the one-way ANOVA and Dunn's multiple comparison test found that the mean value of the bacterial counts was significantly different between the Industrial Zone and Phosphate Berth sites ($p = 0.00704$). However, no significant differences were obtained for the bacterial count values between the two coral species (one-way ANOVA, F (1,16) = 0.617, $p = 0.443$)).

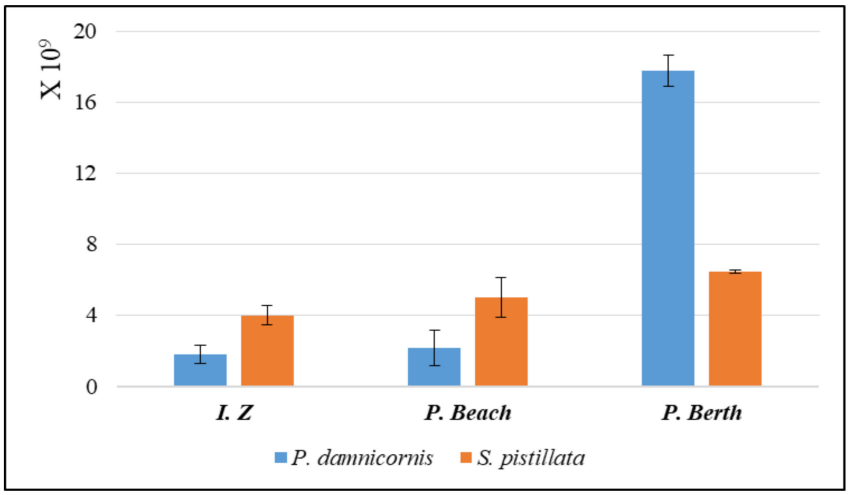

Figure 2. Viable bacterial count associated with the mucus of *P. damicornis* and *S. pistillata* expressed in colony-forming units per mL (CFU/mL). (I.Z: Industrial Zone, P. Beach: Public Beach, P. Berth: Phosphate Berth).

The viable bacterial count of the seawater surrounding both corals, *S. pistillata* and *P. damicornis*, was lower in comparison to that of the mucus (Figure 3). The maximum value was detected at the Phosphate Berth site for *P. damicornis* coral species (1.22×10^6), while the lowest values were obtained from the seawater around both coral species from the Public Beach site. The variations in the bacterial viable count values between the coral mucus and surrounding seawater were significantly different ((F (1,10) = 6.536, $p = 0.028$, one-way ANOVA), post hoc comparison (Dunn's-test, $p = 0.0039$)). Furthermore, significant differences were obtained for a viable bacterial count between the mucus of *S. pistillata* and the surrounding seawater ((F (1,8) = 131.4, $p = 3.3 \times 10^{-6}$, one-way ANOVA), post hoc comparisons (Dunn's-test, $p = 0.0088$)). However, no significant differences were obtained between the mucus of *P. damicornis* and the surrounding seawater (one-way ANOVA, F (1,10) = 4.74, $p = 0.054$).

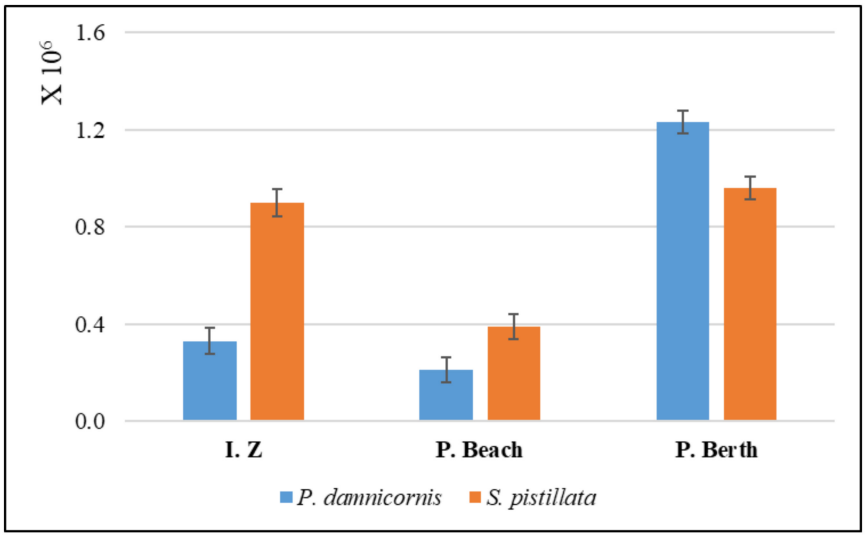

Figure 3. Viable bacterial counts of seawater surrounding *P. damicornis* and *S. pistillata* expressed in colony-forming units per mL (CFU/mL). (I.Z: Industrial Zones, P. Beach: Public Beach, P. Berth: Phosphate Berth).

3.2. Biochemical Identification of Bacteria

A total of 58 different bacterial colonies were isolated from the coral mucus samples from the sampling sites for both coral species based on the unique colonial characteristics of the MA medium. Of them, 22 of the bacteria isolated were obtained from the mucus of *P. damicornis*, whereas the remaining 36 isolated were obtained from *S. pistillata* mucus. The distinct morphological and colony characteristics of the isolated bacteria are shown in Table S2.

Following the morphological and biochemical pathway for the taxonomical identification of the bacterial isolate from *P. damicornis* mucus, 15 isolates were identified at the family level (Figure 4). The bacterial isolates belonged to the classes Gammaproteobacteria (73%), Cytophagia (20%), and Actinomycetia (7%). High variability in Public Beach isolates was detected as compared with those from the other sampling sites (Tables S2 and S4).

Bacterial species belonging to the families *Yersiniaceae* and *Cytophagaceae* were found to be dominant in *P. damicornis* mucus. Bacterial species of the families *Micrococcaceae* and *Pseudomonadaceae* showed the lowest abundance among the sampling sites.

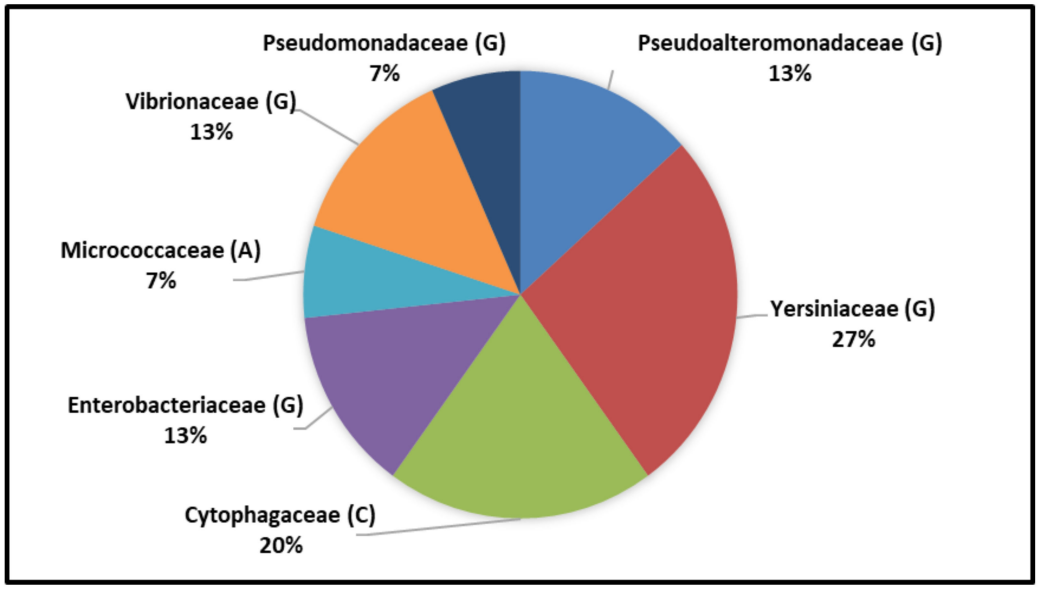

Figure 4. Proportion of coral (*P. damicornis*) mucus bacterial isolates based on the biochemical approach (A: Actinomycetia; C: Cytophagia; and G: Gammaproteobacteria).

Higher diversity of bacterial species was identified from coral *S. pistillata* mucus samples among the sampling sites. The taxonomical identification using morphological and biochemical tests resulted in 26 bacterial isolates (at the family level) (Figure 5). The bacterial isolates were distributed among the classes Gammaproteobacteria (58%), Actinomycetia (23%), Alphaproteobacteria (11%), and Bacilli (8%). The Public Beach site was characterized by higher diversity in comparison with the other sites. The *S. pistillata* mucus bacterial communities were characterized by the dominance of species belonging to the families *Pseudomonadaceae*, *Vibrionaceae*, *Corynebacteriaceae*, and *Yersiniaceae*. The bacterial families from the class Bacilli (*Caryophanaceae* and *Streptococcaceae*) showed the lowest abundance in the *S. pistillata* mucus bacterial community.

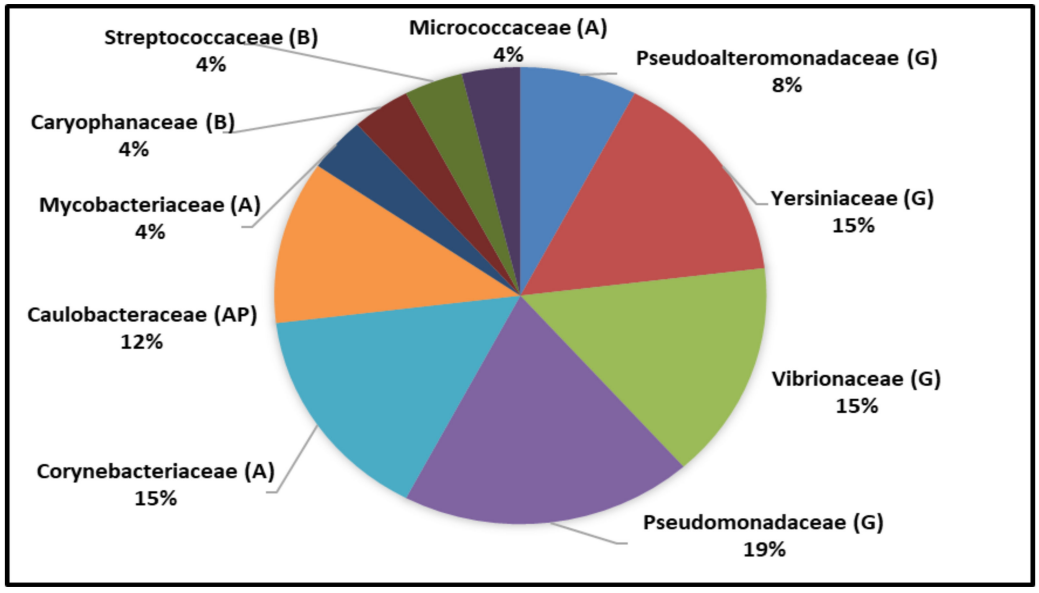

Figure 5. Proportion of bacterial isolates in coral (*S. pistillata*) mucus based on a biochemical approach (A: Actinomycetia; B: Bacilli; AP: Alphaproteobacteria; C: Cytophagia; G: Gammaproteobacteria).

Regarding seawater isolates, a total of 62 bacterial isolates with unique colonial characteristics were obtained from both seawater samples (Tables S3 and S5). Thirty isolates were

obtained from the seawater collected around *P. damicornis*, and 32 isolates from the seawater around *S. pistillata*. From the total isolates, 48 bacterial isolates were identified using a conventional biochemical test (Figure 6). The seawater bacterial community surrounding *P. damicornis* was mainly composed of Gammaproteobacteria (78%), Fusobacteriota (13%), and Bacilli (9%), whereas the *S. pistillata*-surrounding seawater bacterial community was composed of Gammaproteobacteria (84%), Actinomycetia (8%), Fusobacteriota (4%), and Bacilli (4%). More than three quarters of the bacterial isolates from both seawater samples belonged to the families *Pseudoalteromonadaceae*, *Pseudomonadaceae*, and *Yersiniaceae*. The lowest abundances were noticed for the bacterial families *Enterobacteriaceae*, *Nocardiacaea*, and *Actinomycetaceae*.

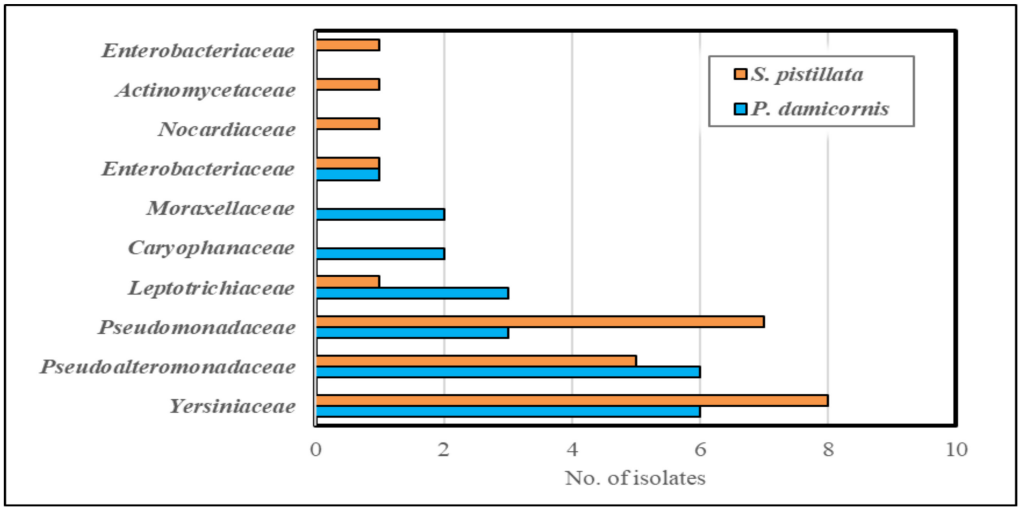

Figure 6. Number of bacterial isolates in seawater surrounding coral based on a biochemical approach.

3.3. Molecular Identification of Bacteria and Phylogenetic Analysis

Among the bacterial isolates that could not be identified by conventional biochemical tests, a total of 17 mucus-associated bacterial isolates were identified at the species level based on 16 S rRNA gene sequencing; among them, 7 and 10 isolates were isolated from the *P. damicornis* and *S. pistillata* mucus, respectively (Table 1).

The Public Beach site was characterized by the highest bacterial diversity. The most common bacterial species isolated from the mucus of *P. damicornis* belonged to the genera *Pseudoalteromonas* and *Psychrobacter*. Interestingly, the species *Pseudoalteromonas* sp. was isolated from the mucus of *P. damicornis* at all sampling sites, whereas the species *Cellulophaga lytica* (family: *Flavobacteriaceae*) was exclusively isolated from samples from the Phosphate Berth site.

Fourteen bacterial isolates from seawater samples around the two coral species were identified using a molecular approach (Table 2). They were divided equally between both coral species. The bacterial species belong to Gammaproteobacteria and Bacilli classes. Bacteria from the genus *Bacillus* were isolated from seawater samples from both Industrial Zone and Phosphate Berth sites. The bacterial strains from the Public Beach site belonged to the Gammaproteobacteria class.

Table 1. Identification of coral mucus-associated bacterial isolates based on 16S rRNA gene identity.

	Sample	Site	Closest Match in GenBank	% Identity	Accession Number
P. damicornis	APm25	I.Z	*Pseudoalteromonas* sp. strain 70410	97.8	KX833144.1
	APm32		*Pseudoalteromonas* sp. strain 70367	97.7	KX889955.1
	BPm9	P. Beach	*Psychrobacter celer* strain Mcap_H2	98.6	KP640590.1
	BPm21		*Pseudoalteromonas* sp. strain 70607	96.9	KY272021.1
	CPm6	P. Berth	*Psychrobacter celer* strain 32	97.2	FJ613610.1
	CPm13		*Pseudoalteromonas* sp. strain 70004	98.0	MF061257.1
	CPm48		*Cellulophaga lytica* strain IMCC34136	96.7	MG456766.1
S. pistillata	ASm14	I.Z	*Pseudoalteromonas* sp. strain NBTE-X3	97.7	MW709811.1
	ASm17		*Vibrio* sp. strain 201705CJKOP-47	96.8	MG309360.1
	BSm20	P. Beach	*Vibrio* sp. Mj76	96.0	GQ455012.1
	BSm24		*Vibrio halioticoli* strain Msp2-1	97.9	MK334316.1
	BSm36		*Bacillus* sp. MML3	99.0	JX847617.1
	CSm16	P. Berth	*Agarivorans* sp. VibC-Oc-065	98.0	KF577091.1
	CSm18		*Psychrobacter celer* strain 32	97.1	FJ613610.1
	CSm34		*Psychrobacter* sp. strain 201705CJKOP-104	96.0	MG309417.1
	CSm37		*Shewanella fidelis* strain 3313	98.0	KY696838.1
	CSm38		*Shewanella* sp. strain MH6	97.6	MN049712.1

I.Z: Industrial Zone, P. Beach: Public Beach, P. Berth: Phosphate Berth.

Table 2. Identification of bacterial isolates from seawater surrounding coral based on 16S rRNA gene identity.

	Sample	Site	Closest Match in GenBank	% Identity	Accession Number
P. damicornis	APw2	I.Z	*Marinomonas aquiplantarum* strain IVIA-Po-183	96.0	EU188446.1
	APw5		*Bacillus thuringiensis* isolate PG05	98.0	EU161995.1
	BPw9	P. Beach	*Acinetobacter schindleri* strain LUH5832	95.7	MG581287.1
	CPw2	P. Berth	*Psychrobacter marincola* strain MTa2-2-1	98.4	MW675164.1
	CPw5		*Bacillus cereus* isolate PGO6	97.9	EU161996.1
	CPw8		*Bacillus firmus* strain C21	96.1	MT457439.1
	CPw4		*Halomonas venusta* strain 0099	98.3	KP236234.1
S. pistillata	ASw1	I.Z	*Bacillus cereus* strain CC2H2P	97.9	KX424371.1
	ASw8		*Bacillus oceanisediminis* strain SH-63	97.5	KX959969.1
	BSw5	P. Beach	*Halomonas venusta* strain 0099	98.3	KP236234.1
	BSw2		*Marinovum algicola* strain ROA150	98.4	MW965560.1
	BSw9		*Vibrio chagasii* strain 3-7	97.8	MN938232.1
	BSw11		*Shewanella fidelis* strain S841	97.0	MK452729.1
	CSw8	P.Berth	*Bacillus horikoshii* strain M2-1	97.7	KF358263.1

I.Z: Industrial Zone, P. Beach: Public Beach, P. Berth: Phosphate Berth.

Phylogenetic analysis of the isolated bacteria from the coral (*P. damicornis*) mucus and the surrounding seawater revealed the presence of two major groups of bacterial taxa, the Gram-positive Bacillota (also called Firmicutes) and the Gram-negative Gammaproteobacteria (Figure 7). Phylogenetic analysis of the coral mucus and seawater strains showed that there were 10 strains clustered within the Gammaproteobacteria group belonging to several Pseudomonadales, Alteromonadales, and Oceanospirillales bacterial orders with 96–99% similarity to sequences in the NCBI databases. Gram-positive *Bacillus* strains were clustered in a distinct cluster. BLAST analysis showed that the bacterial strain BPw9 was a member of the Gammaproteobacteria, with the lowest similarity percentage (95.7%) with *Acinetobacter schindleri* (accession number MG581287.1). A single cluster was noticed for *Psychrobacter* species with similarity of >97% with the BLAST database.

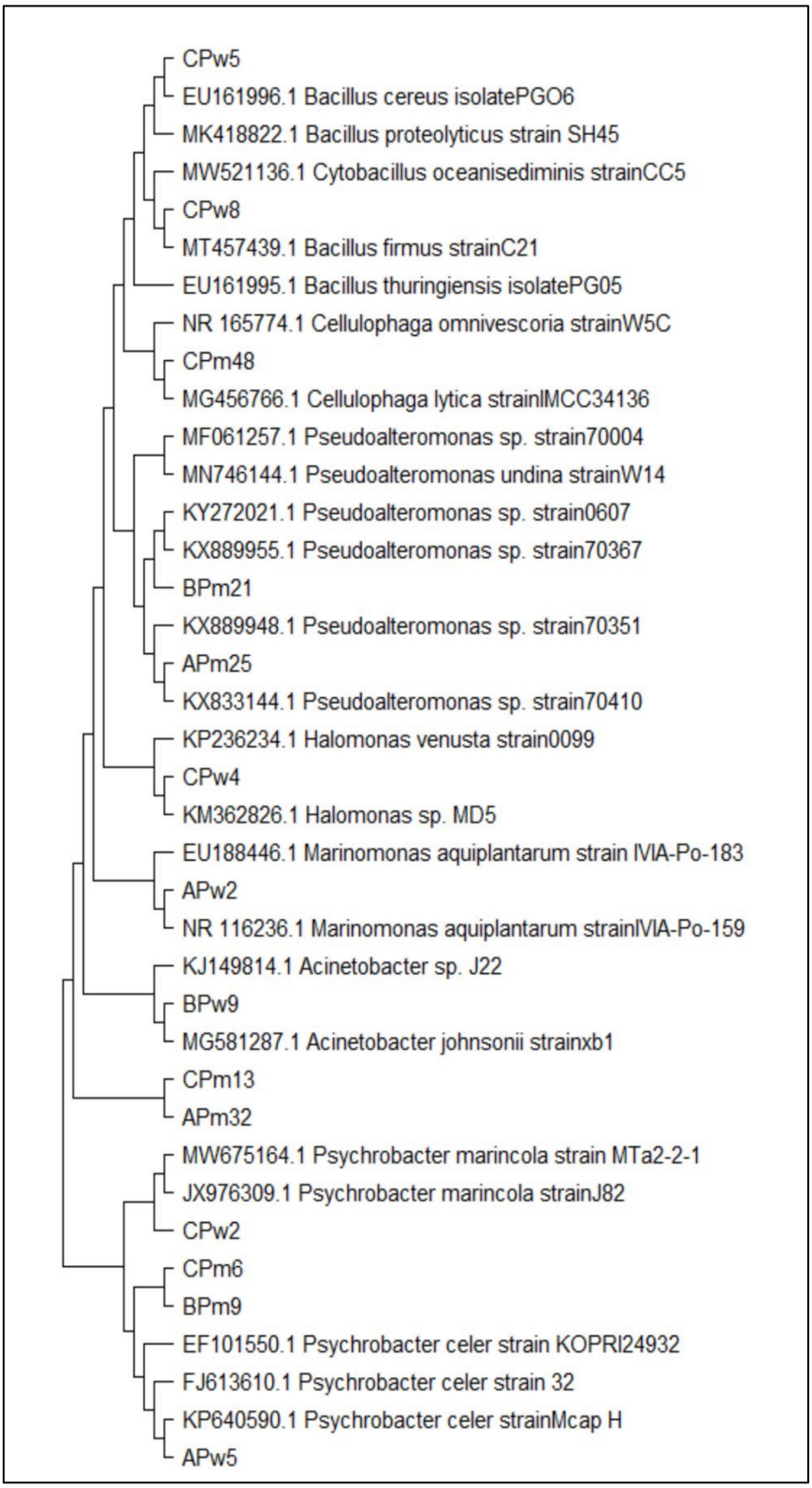

Figure 7. Phylogenetic tree for bacterial species associated with *P. damicornis* mucus and the surrounding seawater. Phylogenetic analyses were conducted in MEGA11. There were a total of 2303 positions in the final dataset. A: Industrial Zones, B: Public Beach, C: Phosphate Berth, P: *P. damicornis*, m: mucus, w: seawater.

Similarly, two major groups of bacterial domains, Gram-positive Bacillota and the Gram-negative Gammaproteobacteria, were obtained from the phylogenetic analyses of bacterial isolate sequences from the coral *(S. pistillata*) mucus and the surrounding seawater (Figure 8). From the four bacilli isolates cluster, one isolate was isolated from *S. Pistillata* mucus (BSm36), which had 99% similarity with *Bacillus* sp. MML3 (accession number JX847617.1). A total of 13 strains were clustered within the Gammaproteobacteria group belonging to several Pseudomonadales, Alteromonadales, Oceanospirillales, and Vibrionales bacterial orders with 96–98.4% similarity with NCBI database sequences. Each bacterial member of the Gammaproteobacteria showed a unique cluster with the same phylogenetic distance, except for the species *Marinovum algicola* (family: *Oceanospirillaceae*) (BSw2), which was isolated from the Public Beach site seawater; this strain had a distinct cluster.

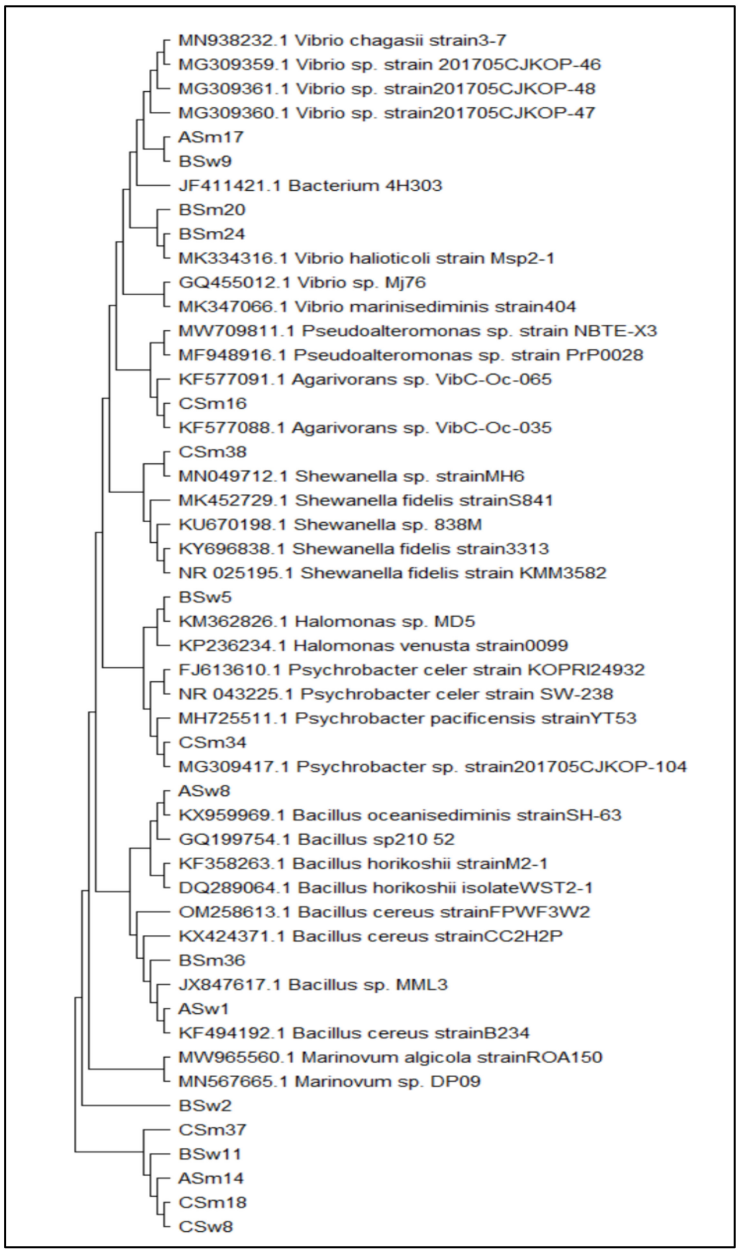

Figure 8. Phylogenetic tree for bacterial species associated with *S. pistillata* mucus and the surrounding seawater. Phylogenetic analyses were conducted in MEGA11. There were a total of 1739 positions in the final dataset. A: Industrial Zone, B: Public Beach, C: Phosphate Berth, S: *S. pistillata*, m: mucus, w: seawater.

3.4. Distribution of Shared and Non-Ubiquitous Bacterial Isolates

The intersection among the coral species and sampling sites through Venn diagrams (Figure 9a) showed that *S. pistillata* exhibited the highest number of non-ubiquitous bacterial genera (10 genera). The diagram showed that bacterial isolates belonging to seven genera were commonly distributed in the mucus of both coral species, while bacterial species belonging to three genera—*Cellulophaga*, *Cytophaga*, and *Klebsiella*—were exclusively distributed in the mucus of *P. damicornis*.

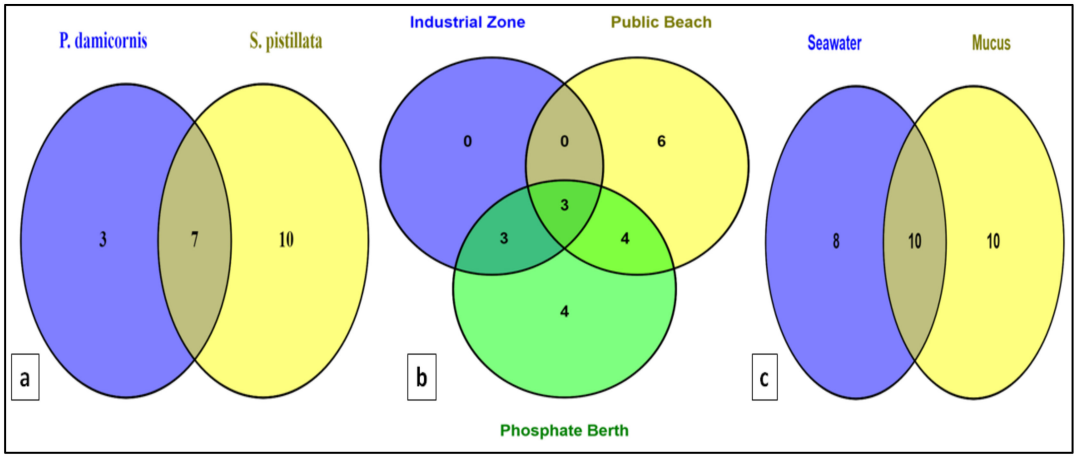

Figure 9. Distribution of shared and non-ubiquitous bacterial isolates (**a**) between coral species, (**b**) between sampling sites, and (**c**) between seawater and coral mucus.

Bacterial species belonging to the three genera *Brevundimonas*, *Pseudomonas*, and *Vibrio* were shared between all sampling sites (Figure 9b). Interestingly, no unique bacterial species were present at the Industrial Zone site. However, bacterial species belonging to six genera—*Bacillus*, *Klebsiella*, *Mesophilobacter*, *Mycobacterium*, *Photobacterium*, and *Planococcus*—were exclusively distributed at the Public Beach site. SIMPER analysis indicated that the lowest similarity between the bacterial communities was obtained for the Industrial Zone and Phosphate berth site (26.2%), where the species of the bacteria genera *Pseudoalteromonas*, *Serratia*, *Pseudomonas*, *Cytophaga*, *Bacillus*, *Cellulophaga*, *Streptobacillus*, *Vibrio*, and *Yersinia* contributed to ca. 60% of the dissimilarity between the two sites. By comparing the distribution of the bacterial communities between coral mucus and the surrounding seawater, the Venn diagram (Figure 9c) showed that bacterial species from 10 genera were shared between the two matrices. SIMPER analysis revealed that high dissimilarity was obtained between the coral mucus and seawater bacterial community (76.7%). The species of bacterial genera belonging to *Serratia*, *Pseudomonas*, *Pseudoalteromonas*, *Vibrio*, *Bacillus*, *Yersinia*, *Cytophaga*, and *Streptobacillus* contributed to ca. 51% of the dissimilarity between the coral mucus and seawater bacterial communities.

3.5. Distribution of Coral Mucus and Seawater Bacterial Communities

The distribution of the coral mucus and surrounding seawater bacterial communities among the sampling sites were elucidated by principal coordinates analysis (PCoA) (Figure 10). The first two coordinates of PCoA expressed 55% of the total variations between the bacterial communities. The coral mucus bacterial communities' samples were clustered together, whereas the seawater bacterial community samples were clustered in a distinct cluster. Interestingly, samples from the Industrial Zone site were clustered together away from the other sampling sites.

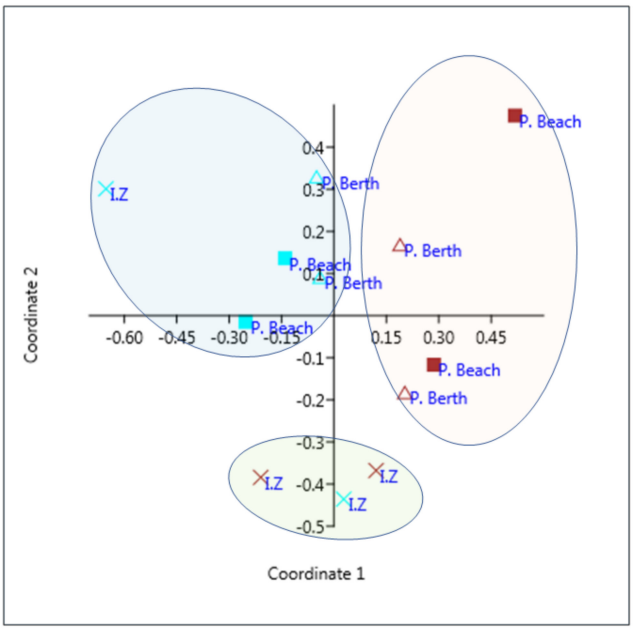

Figure 10. Principal coordinates analysis plot (PCoA) based on a Euclidean distance matrix calculated of the mucus bacterial community and the surrounding seawater. (Brown marks represent coral mucus samples and blue marks represent seawater samples) [X: Industrial zone samples, ■: Public Beach samples, Δ: Phosphate Berth samples].

3.6. Antimicrobial Activity of Coral Mucus and Seawater Bacterial Isolates

Out of the 120 bacterial isolates that were examined for antibacterial activity, 10 isolates exhibited antimicrobial activities against at least one indicator strain (8.6% of mucus and 8.0% of seawater) (Table 3). Strain BPw9 was found to be active against *P. aeruginosa*, *S. marcescens*, and *S. aureus* (inhibition zones: 16, 13, and 13 mm, respectively). Six strains exhibited activity against *S. marcescens* ATCC27117. The strain CPw4 only showed activity against *M. luteus* ATCC9341 (inhibition zone, 12 mm). The highest antimicrobial activity was noticed for the strains against *P. aeruginosa* ATCC13048, with the highest diameter in the zone of inhibition.

Table 3. Antibacterial activity of isolated strains from the coral mucus and the surrounding seawater.

	Mucus						Seawater			
	ASm14	**BPm21**	**BSm24**	**BSm36**	**CSm16**	**APw2**	**ASw8**	**BPw9**	**CPw3**	**CPw4**
S. marcescens	7.4 (1.3) *	8.0 (1.1)	-	6.2 (1.4)	16.2 (2.2) *	9.1 (1.5)	-	19.4 (1.6)	-	-
P. aurgenosa	-	-	24.1 (3.2)	-	23.4 (2.1) *	-	-	25.0 (3.6)	-	-
S. aureus	9.3 (1.2) *	-	-	-	-	-	15.0 (2.2)	20.2 (2.3)	20.6 (2.1)	-
M. luteus	-	-	-	-	-	-	-	-	-	17.4 (2.5)

* Isolate grown in media for 72 h; all other isolates grown in marine broth for 48 h.; - Zone of inhibition (mm (SD)).

4. Discussion

The study of microbial communities under a range of anthropogenic pollutants along the Jordanian coast of the Gulf of Aqaba revealed an influence of prevailing anthropogenic pressures on the microbial communities associated with the mucus and surrounding seawater of the corals *P. damicornis* and *S. pistillata*. The effect of anthropogenic pollutants was clearly evidenced by the prevalence of bacterial species that are known to be related to coral diseases, such as white plague-like, pink-blue spot syndrome, and dark spots [45]. These bacterial communities may originate from the pollutants discharged into seawater

from the adjacent environment and the industrial activities on the coastline of the Gulf of Aqaba [46]. Intriguingly, the experiment by Ziegler et al. [47] found that the coral *Acropora hemprichii* harbored a highly flexible microbiome that differed in response to the level of anthropogenic impact for the transplanted corals. However, *Pocillopora verrucosa* (Ellis and Solander, 1786) remained remarkably stable.

The average viable bacterial count from coral mucus for both studied corals ranged between 10^7 and 10^9 CFU/mL. These concentrations of bacteria in mucus agreed with Omry and Eugene [34], who investigated the number of bacteria in the mucus of the Mediterranean coral *Oculina patagonica* de Angelis D'Ossat, 1908; they found that the average viable bacterial count was 3×10^8. Furthermore, the viable bacterial count values from mucus from the studied sites is consistent with the results obtained by Jaber [48] who studied the bacterial communities associated with the corals *S. pistillata* and *Galaxea fascicularis* (Linnaeus, 1767) at the Marine Science Station (MSS) in the Gulf of Aqaba (Jordan). This high number of bacteria in coral mucus could be related to the sufficient nitrogen and phosphorus (eutrophication) in the mucus that support the growth of bacteria [49]. However, a lower bacterial count was recorded for seawater surrounding the studied coral species. The oligotrophic condition of Gulf of Aqaba seawater may be attributed to the inhibition of bacterial growth in the water column [50].

The heterotrophic cultivable bacterial communities associated with the mucus of these corals consisted of the bacterial phyla Proteobacteria (82%), Actinomycetia (7%), Cytophagia (6%), and Bacilli (6%). The bacteria diversity was similar to that of those isolated from the mucus of four coral species present along the Brazilian coast [51], where a higher abundance of Proteobacteria was also detected. Indeed, this phylum has already been detected in association with the mucus of the coral *Fungia scutaira* Lamarck, 1801, which was studied by Lampert et al. [35] in the Red Sea area. In the coral holobiont, the phylum Proteobacteria has shown antimicrobial properties and the ability to induce larval settlement [52], suggesting an important role for this phylum in protecting coral health. Gammaproteobacteria (76%) and Alphaproteobacteria (6%) were the most dominant classes. Gammaproteobacteria was the most abundant group of cultivable bacteria in *Mussismilia hispida* (Verrill, 1901) and *Madracis decactis* (Lyman, 1859) coral mucus samples [51]. The high proportions of these bacteria in the coral mucus could be indicative of the importance of these bacteria in the coral holobiont.

The use of coral-surrounding seawater bacterial community data, together with coral mucus data, may enhance our ability to evaluate the effect of anthropogenic stressors and environmental changes more holistically. For example, the seawater bacterial communities could change in response to temperature fluctuations [50], which is consequently correlated with coral bleaching [53]. The seawater bacterial communities were predominated by Gammaproteobacteria (76%), Bacilli (15%), Fusobacteriia (6%), and Actinomycetia (3%) classes. These findings disagreed with the previous results obtained by Kooperman et al. [54], where they studied the bacterial association between two coral species and the surrounding seawater in the Red Sea. They found that the most abundant group in seawater samples was cyanobacteria (30%), whereas the Gammaproteobacteria group accounted for only 5%. Consistent with our results, Jorge et al. [55] found that Gammaproteobacteria was the most abundant group in the seawater around the coral *Montastrea cavernosa* (Linnaeus, 1767) in the Caribbean.

Spatial variations among the coral mucus-associated bacterial communities were verified between the sampling sites from the Gulf of Aqaba (Figure 10). The distinct cluster of Industrial Zone samples located in the southern part of the Gulf of Aqaba might be correlated with the higher pollutant levels (e.g., metals [30]). *Mesophilobacter* sp. (which was isolated exclusively from the Public beach) showed antagonistic activity against 17 multi-drug-resistant pathogens, including bacteria and fungi [56]. The sewage discharge and swimming activities may have been attributed to the presence of this species in the seawater column [57]. The emission of hydrocarbons from touristic boats may be related to the enrichment of *Planococcus* bacteria. The genus *Planococcus* has been reported

to have the ability of aromatic hydrocarbon degradation with biosurfactants/bioemulsifiers secretion [58]. Remarkably, some species of the genus *Photobacterium* (*Photobacterium rosenbergii*) that were cultivated at the Public Beach site are known to be associated with coral bleaching [59]. Similarly, the level of putative pathogenic bacteria *Klebsiella* was higher in diseased stony corals (*Acropora Cytherea* (Dana, 1846)) from India [60]. These bacterial species may be originated from swimming activities and sewage discharge at the Public Beach. Some of the common bacterial isolates in the Industrial Zone and Phosphate Berth sites belonged to the species *Pseudoalteromonas*, which were found to have the ability to convert different forms of aromatic compounds into the corresponding catechol, which, consequently, may be introduced into the bioremediation of chemically contaminated marine environments [61]. The dominance of this bacterial species at these sites might be correlated with oil and aromatic compound contamination. Furthermore, *Cytophaga* sp. was previously isolated from coral surfaces infected with black band disease [55]. The presence of this species can be related to the effect of coastal industrial activities that discharge at these sites on coral health.

Species from three ubiquitous bacterial genera were presented among the sampling sites (*Brevundimonas*, *Pseudomonas*, and *Vibrio*). The bacterial communities associated with three species of *Acropora* from Orpheus Island that comprised the greatest portion of the clone libraries belonged to *Brevundimonas* sp. (4–22%), which are possible candidates for investigation in coral nutrient cycling or the production of antimicrobial properties [62]. Bacteria from the genus *Pseudomonas* are among the mucus' core microbiome and constituted the most abundant taxa in the corals from the Red Sea and Persian/Arabian Gulf [63]. Furthermore, Lalucat et al. [64] reported that *Pseudomonas* is one of the highest taxonomic clusters of known denitrifying bacteria. *Pseudomonas* sp. are considered to be the most active denitrifying heterotrophic bacteria in the environment, which include metal cycling and the degradation of biogenic and xenobiotic compounds [64]. Interestingly, some coral pathogens belonging to *Vibrio*, (ex., *Vibrio coralliilyticus*, a Red Sea pathogen of *P. damicornis* [65]) increase their efficiency and motility behaviors with rising seawater temperatures [66], and the higher abundance of these microbes among sampling sites may explain the increased prevalence of coral disease post-bleaching [45,67].

Bacterial species-specific associations were noticed between the mucus coral species, such as *Cytophaga* sp., which was exclusively present in association with *P. damicornis* mucus. Arboleda and Reichardt [68] demonstrated that diseased *P. damicornis* was dominated by *Cytophaga* (Bacteroidota) in the Lingayen Gulf, Philippines. Moreover, bacteria from the genus *Pseudomonas* were found to be highly associated with the Red Sea coral *S. pistillata* [69]. *Corynebacterium* sp. was one of the eight genera with relatively high abundance and was detected in $\geq$80% samples of *S. pistillata* during a long-term survey conducted by Yang et al. [70] in Taiwan. Intriguingly, during a study on the influence of species specificity on bacteria associated with the coral *S. pistillata* in Taiwan, Mei-Jhu et al. [71] found that the largest operational taxonomic unit (OUT) belonged to *Bacillus* sp. (ex. *Bacillus cereus* or *Bacillus thuringiensis*), appearing mainly in winter samples, which was the same sampling period of our study. Overall, the variations in the mucus associated-bacterial communities between the coral species *P. damicornis* and *S. pistillata* suggest the different degrees of coral holobiont flexibility. These potentially represent differences in the underlying strategy employed by the two species to cope with environmental stressors [47].

Remarkably, the seawater samples surrounding the corals had a distinct bacterial clustering (Figure 10) characterized by the presence of non-ubiquitous bacteria, including representatives of the Gammaproteobacteria, Actinomycetia, Bacilli, and Fusobacteriia classes. Unlike the results obtained by Osman et al. [72] regarding the stability of microbes in reef-associated seawater affected by anthropogenic development in the Red Sea, we found variations in the bacterial communities among the seawater samples surrounding the corals at the different sites. Intriguingly, the bacterium *Marinobacter*, which was present exclusively in seawater, was found to be the predominant oil-degrading bacteria in polluted seawater of the Yellow Sea, China [73]. Rajeev et al. [74] noticed that the thermal

discharge-impacted coastal areas were overrepresented by several potential pathogenic bacteria (e.g., *Acinetobacter*) and other native marine bacterial genera (e.g., *Marinobacter* and *Halomonas*). *Streptobacillus* sp., for instance, which was exclusively isolated from seawater samples, was related to seawater disease in farmed Atlantic salmon [75].

Several bacteria from the coral holobiont are known to produce antimicrobial agents for survival and defense purposes. Both coral mucus-associated bacterial samples and coral-surrounding seawater bacterial samples were screened for the presence of antimicrobial activity. Among these, ten isolates (8.3%) showed antimicrobial activity toward at least one *ATCC*-tested bacteria. This percentage is slightly higher than the previous study that showed that 5.77% of cultivable bacteria isolated from the mucus of *Oculina patagonica* produced antimicrobial activity [76]. However, isolates from the coral *A. palmata* showed a higher percentage (20%) of antibiotic producers [11]. This can be related to the assay method, as well as the species and the number of indictor bacterial strains used in screening.

The antimicrobial screening test revealed that high bacterial diversity was able to inhibit the growth of a *S. marcescens* strain. Many strains of *S. marcescens* are known as opportunistic pathogens responsible for white pox disease, causing coral tissue necrosis [77]. These pathogenic bacteria colonize the coral mucus layer, utilizing the complex polymers of the mucus as a carbon source [78,79]. Nissimov et al. [76] suggested that the commensal bacteria present in the coral mucus can prevent the complete establishment of *S. marcescens* by niche occupation or antimicrobial production. These support the hypothesis that the members of the mucus microbiome may support the defense mechanism of corals. The bacteria *Vibrio halioticoli* and *Acinetobacter schindleri* showed the highest inhibitive effects against the pathogenic indicator bacteria *P. aurgenosa*, indicating the production of bioactive materials to inhibit the growth of specific marine microbial competitors [76]. All of the above results suggest that the interactions of the coral holobiont could be diverse and complicated, where different coral bacteria may contribute differently to the protection of the coral from marine pathogens.

5. Conclusions

The findings of this study obviously highlight the variations in bacterial communities among the studied mucus coral samples and among contaminated sampling sites. Habitat specificity was observed among the coral mucus-associated bacterial communities and the surrounding seawater microbes, confirming the compositional variability of microbial communities. The diversity and spatial variations of bacterial communities represented by clustering may reflect the response of bacterial communities to local environmental stressors. Habitat specificity contributes to the overall diversity of microbial communities, highlighting the importance of holistic studies that focus on microbial interactions across the coral reef ecosystem.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jmse10070863/s1, Table S1: Sources and level of anthropogenic pressure in the sampling site of the Gulf of Aqaba [29–32,80–82], Table S2: Morphological characterization of mucus bacterial isolates, Table S3: Morphology and characterization of seawater bacteria isolates, Table S4: Biochemical and physiological characterization of bacterial isolates from mucus, Table S5: Biochemical and physiological characterization of bacterial isolates from seawater, Table S6: Phylogeny of bacterial species from coral mucus and surrounding seawater.

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