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Bacterial Dynamics in Newly Settled *Acropora kenti*: Insights From Inoculations With Individual Probiotic Candidates

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ABSTRACT

Bacterial probiotics hold promise for enhancing coral health and stress resilience; however, their application to coral juveniles, which are critical for restorative aquaculture, remains underexplored. Here, we show that the microbiome of newly settled *Acropora kenti* is amenable to manipulation, with individual probiotic candidates inducing distinct microbiome responses that differed in their stability after the final inoculation. Three of the eight tested strains, *Halomonas smyrnensis*, *Endozoicomonas acroporae* and *Roseivivax lentus*, remained prevalent in the microbiome at least 5 days post-inoculation. Notably, aggregates of *Endozoicomonas* cells were observed exclusively in the corresponding treatment, marking the first report of coral-associated microbial aggregates (CAMAs) in early life stage acroporids and the first induced through bacterial manipulation. In the *Pseudoalteromonas rubra* treatment, the inoculum was detected at low relative abundance but induced a microbiome shift that persisted beyond the final inoculation. While no significant coral health benefits were observed in this short 17-day experiment, the *Ruegeria arenilitoris* strain unexpectedly induced tissue regression and mortality. The described experimental system offers a systematic approach for tracking bacterial dynamics in newly settled corals, facilitating the identification of potentially beneficial strains and the exclusion of harmful ones to inform the design of probiotic consortia for coral aquaculture.

1 | Introduction

Scleractinian corals are essential marine ecosystem engineers, building reefs that support immense biodiversity and play critical roles in global ecological processes, including nutrient cycling and carbon sequestration (Bellwood and Hughes 2001; Brandl et al. 2019). Coral reefs also contribute significantly to local and global economies through tourism, shoreline protection and fisheries (Costanza et al. 2014; Putnam et al. 2017). However, these ecosystems are under increasing threat from global pressures, particularly rising sea-surface temperatures, which have led to mass bleaching events and high coral mortality (Hughes et al. 2018). These alarming declines have prompted active reef restoration initiatives, alongside efforts to mitigate global warming and reduce localised impacts (Hein et al. 2021).

The first two authors contributed equally to this work.

The health and resilience of scleractinian corals are closely tied to their microbiome, which includes algal endosymbionts from the family Symbiodiniaceae and diverse coral-associated bacteria (Bourne et al. 2016). With these bacteria performing vital roles such as nutrient production and cycling, immune modulation and antimicrobial activity, disruptions to the microbiome can compromise coral health (Ritchie 2006; Thompson et al. 2014; Bourne et al. 2016). Manipulating coral microbial communities has emerged as a promising strategy for enhancing coral resilience to heat stress and reducing disease symptoms (Rosado et al. 2019; Santoro et al. 2021; Li et al. 2023; Ushijima et al. 2023; Xu et al. 2024).

For instance, putative beneficial bacteria selected by Rosado et al. (2019) mitigated bleaching and disease in *Pocillopora damicornis* under heat stress, while Santoro et al. (2021) demonstrated improved photosynthetic performance and reduced mortality in heat-stressed *Mussismilia hispida* treated with a probiotic consortium. Decreased susceptibility to heat stress was also achieved in *P. damicornis* through inoculations with a reactive oxygen species (ROS)-scavenging bacteria, which appeared to reduce ROS levels (Tang et al. 2024). Similarly, administering a single bacterial strain alleviated thermal stress-induced bleaching in *P. damicornis* (Li et al. 2023), and arrested the progression and transmission of stony coral tissue loss disease in *Montastraea cavernosa* (Ushijima et al. 2023). However, it often remains unclear whether the observed benefits arise from direct microbially mediated activities or are merely a result of nutritional supplementation provided by the probiotics (Doering, Maire, et al. 2023). Additionally, while these studies underscore the potential of microbiome manipulation to improve the health and resilience of adult corals, evidence supporting its application in newly settled corals is still limited.

Sustainable coral aquaculture is crucial for supplying stock to support reef restoration strategies, and probiotics have been proposed as a tool to enhance the growth, health and resilience of coral juveniles produced in these closed systems (Randall et al. 2020; Peixoto et al. 2021; Thatcher et al. 2022; Banaszak et al. 2023). Newly settled corals (single polyps), also known as spat, are of particular interest because some coral species acquire most, if not all, of their microbiome horizontally from the environment (Apprill et al. 2009; Sharp et al. 2010; Bernasconi et al. 2019; Damjanovic, Menéndez, et al. 2020). This high microbiome flexibility in early life stages (Littman et al. 2009; Lema et al. 2014) offers a unique window for microbiome manipulation. For example, Damjanovic et al. (2017) demonstrated that inoculating coral larvae with mucus-associated bacteria could shift microbiome communities during development. In another study, the microbiomes of *Acropora kenti* and *Platygyra daedalea* spat were manipulated using the same bacterial consortium, revealing species-specific responses to bacterial inoculations (Damjanovic et al. 2019). Despite these advances, the interactions between individual bacterial strains and coral spat, as well as their specific effects on coral physiology, remain poorly understood.

For this study, eight putatively beneficial bacteria were selected from a culture collection of bacteria isolated from healthy adult colonies of *A. kenti* (Table 1). Belonging to the families Endozoicomonadaceae, Halomonadaceae, Oceanospirillacea, Pseudoaltermonadaceae, Roseobacteraceae and Stappiaceae, the strains were selected based on the frequent detection of these bacterial families in coral microbiomes (Huggett and Apprill 2018; O'Brien et al. 2021; Sweet et al. 2021) and because members of the corresponding genera or families have been proposed or successfully applied as probiotics in corals (Peixoto et al. 2017; Rosado et al. 2019; Dungan et al. 2021; Doering, Tandon, et al. 2023; Ushijima et al. 2023;

TABLE 1 | Treatments applied to *A. kenti* spat, including the identity of each bacterial strain as determined by 16S rRNA gene sequencing (Sweet et al. 2021).

Treatment	Inoculum strain identification	Average rel. ab.		Wilcoxon *W*	*p*
		Day 13, $n=5$	Day 17, $n=5$		
Placebo control	—	—	—	—	—
Killed *E. coli*	*Escherichia coli*[a]	15.30%	1.85%	23	0.0317
Pseudovibrio	*Pseudovibrio denitrificans* 2-ATT-B1	23.50%	3.80%	25	0.0079
Ruegeria	*Ruegeria arenilitoris* 2-ATT-W2	51.20%	NA	NA	NA
Neptunomonas	*Neptunomonas* sp. 2-ATT-W3	17.50%	1.10%	25	0.0079
Halomonas	*Halomonas smyrnensis* 2-ATT-W4	60.10%	42.10%	23	0.0317
Endozoicomonas	*Endozoicomonas acroporae* 2-ATT-W5	30.20%	57.00%	5	0.15
Roseivivax	*Roseivivax lentus* 3-ATT-3	59.40%	59.00%	13	1
Pseudoalteromonas	*Pseudoalteromonas rubra* ATM06	0.59%	0.27%	20	0.15
Thalassobius	*Thalassobius mediterraneus* AT-O-15	38.70%	1.70%	25	0.0079
V. coralliilyticus	*Vibrio coralliilyticus* P1[b]	8.10%	2.70%	23	0.0317

Note: For each treatment, the average relative sequence abundances (rel. ab.) of inoculum-specific ASVs are provided for spat collected 1 and 5 days after the last inoculation (Days 13 and 17, respectively). Temporal changes in relative sequence abundance were investigated using the Wilcoxon rank-sum test, with the statistic (W) and p value presented. No data are available for the *Ruegeria* treatment on Day 17 due to high spat mortality, which prevented sampling.
[a]TOPO cloning kit, Invitrogen, United States.
[b]Sussman et al. (2008).

Xu et al. 2024) or in aquaculture of other species (Fjellheim et al. 2010; Kesarcodi-Watson et al. 2010, 2012; Domínguez-Borbor et al. 2019). In vitro assays conducted prior to this study (Table S1) further supported their selection by identifying putative beneficial traits, including antibacterial activity against the coral pathogen *Vibrio coralliilyticus*, digestive enzyme production and biofilm formation. These traits are potentially advantageous in high-density aquaculture settings: antibacterial activity and biofilm formation may support disease resistance through antimicrobial compound production and niche occupation, while digestive enzyme activity could enhance coral growth by increasing nutrient availability.

The present study is the first to investigate the influence of individual probiotic candidates on the early microbiome, growth and health of coral spat. Using a rigorously controlled system in six-well plates, the microbiome and host responses of *A. kenti* spat after inoculation with individual bacterial strains were assessed up to 5 days following the final dose. Bacterial community dynamics were studied by 16S rRNA gene metabarcoding and fluorescence in situ hybridisation, while coral health was assessed by Symbiodiniaceae density, coral basal disk area and activity of phenoloxidase, an enzyme in the melanin synthesis pathway, as a proxy for coral immune response (Palmer, Bythell, and Willis 2012; Van De Water et al. 2015). This short-term, 17-day experiment served as an initial screening tool to effectively identify promising probiotic candidates for *A. kenti* spat and discard unsuitable ones. Together with cross-compatibility testing of individual candidates, the results of this study informed the strategic selection of strains for future development and testing of a multi-strain probiotic consortium aimed at enhancing aquaculture production of juvenile *A. kenti*.

2 | Experimental Procedures

2.1 | Experimental Design

Adult *A. kenti* (formerly *Acropora tenuis*) colonies were collected from the fringing reefs around Magnetic Island (19°7′43.442″ S 146°52′40.076″ E) in October 2021 (GBRMPA permit G21/45348.1). The colonies were transported to the Australian Institute of Marine Science (AIMS) and maintained in the National Sea Simulator for spawning. Six-day-old larvae were settled in a biosafety cabinet onto 180 sterile tissue culture (TC)-treated six-well plates (CLS3516, Corning, United States) on Day 0 ($n = 6$ larvae per well). Each well was pre-filled with 5 mL of autoclaved, 0.22 μm filtered seawater (FSW) and contained a 5 mm^2 chip of autoclaved crustose coralline algae (CCA, species *Porolithon onkodes*) to induce settlement (Abdul Wahab et al. 2023; Randall et al. 2024). CCA chips and any attached larvae or spat were discarded using sterile forceps 24 h after larvae were added. Plates with poor settlement rates (< 20%) were discarded, while the remaining plates were categorised as high (> 80%), medium (60%–80%) or low (20%–40%) settlement. Plates from each category were then randomly assigned to one of 11 treatments ($n = 12$ plates per treatment) ensuring a similar number of spat and consistent settlement distribution across treatments.

Throughout the experiment, plates were incubated at 28°C under lights providing 40 $\mu mol\, m^{-2} s^{-1}$, following a 12-h light:dark cycle. To reduce water evaporation and prevent cross-contamination, replicate plates were stacked in sets of three inside sealed plastic bags ($n = 6$ plates per bag, two bags per treatment). Plates were rotated in bags every 2 days to ensure uniform light exposure. Handling of plates, including opening bags and water changes, was performed in a biosafety cabinet using sterile equipment. Once settlement was complete, the CCA chips were removed with sterile forceps and the water in each well (5 mL FSW) was changed every 2–3 days (Figure 1).

Two days post-settlement, all wells were inoculated with Symbiodiniaceae subclade *Cladocopium* C1 (AIMS Symbiont Culture Facility ID: SCF055-01.10) at a final density of 20,000 cells mL^{-1} (Quigley et al. 2020). The spat were then subjected to one of 11 treatments: four repeat inoculations with one of eight probiotic candidates, a putative coral pathogen (*V. coralliilyticus* P1), heat-killed *Escherichia coli*, or washed culture medium (placebo control) (Table 1). Spat were sampled before the Symbiodiniaceae inoculation on Day 2, before the first bacterial inoculation on Day 4, and one and 5 days after the final bacterial

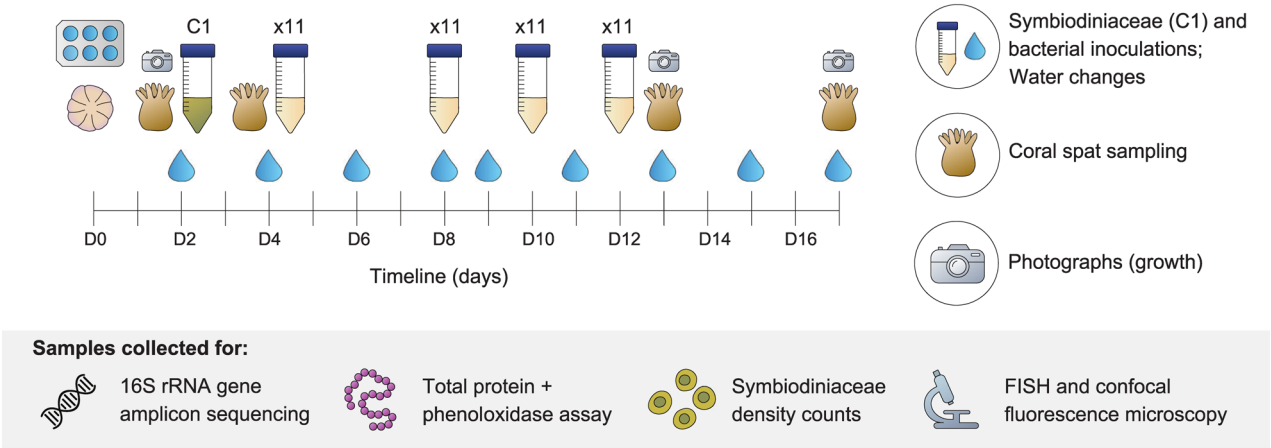

FIGURE 1 | Overview of the experimental timeline with details of settlement, inoculations, water changes and sampling indicated on the layout. D, day of the experiment, with D0 being the settlement of spat in six-well plates.

inoculation (Days 13 and 17, respectively) to assess treatment effects (Figure 1).

2.2 | Bacterial Culturing and Inoculations

The probiotic candidates tested in vivo were previously isolated from *A. kenti* adults using a minimal basal agar medium enriched with methionine and ammonium chloride, and identified by 16S rRNA gene sequencing (Table S1; Sweet et al. 2021; Kuek et al. 2022).

One day before each inoculation, bacterial colonies grown on marine agar (MA; Difco Marine Agar 2216, Becton Dickinson, United States) were transferred into duplicate 10 mL solutions of marine broth (MB; Difco Marine Broth 2216, Becton Dickinson) and incubated overnight at 28°C with shaking at 180 rpm. The resulting cultures for each strain were pooled, centrifuged at 5200 ×*g* for 10 min at 10°C to pellet the cells, washed and resuspended in FSW. Cell concentrations were determined using a haemocytometer (Neubauer chamber, BlauBrand, Germany). Heat-killed *E. coli* cells were prepared by incubating washed cells on a heat block at 80°C for 10 min. This treatment served as a control for possible nutritional effects and non-specific responses to microbial biomass or cell components, without introducing confounding signals from host-associated bacteria, as *E. coli* is not naturally found in corals. Further, the use of heat-killed *E. coli* provided a defined baseline for detecting dead cells via 16S rRNA gene metabarcoding and enzyme activity assays. The placebo control consisted of clean culture medium (10 mL) processed identically to the inoculated cultures. Bacterial and control treatments (Table 1) were administered using a multi-channel pipette to a final density of 2×10^7 cells mL^{-1} on Days 4, 8, 10, and 12 (Figure 1). Because individual bacterial strains differed in culture density, treatment-specific volumes (ranging from 23.2 to 298 μL across all inoculation events) were added to reach the target cell concentration (Table S2). The volume of the placebo control to dispense in each well was calculated as the average volume of all other treatments.

2.3 | Sample Collection

For each treatment, coral spat were randomly sampled from wells across multiple plates to minimise potential bias from variation in spat number per well. The spat were carefully detached using pipette tips pre-cut at an angle and transferred to a sterile FSW bath with Storkbill forceps (Australian Entomological Supplies, Australia) to remove loosely associated microorganisms. After being gently tapped dry, spat designated for DNA extractions ($n = 5$ pools of five spat per treatment) and protein extractions ($n = 3$ pools of 20 spat per treatment) were placed into sterile cryovials and immediately transferred to −80°C. In addition to coral spat, 300 μL of resuspended Symbiodiniaceae culture ($n = 2$) and 300 μL of resuspended and pooled bacterial cultures ($n = 2$) were sampled as positive controls for amplicon sequencing. The volumes of the individual bacterial cultures were adjusted to a final concentration of 2×10^7 cells mL^{-1} per strain. In addition to serving as sequencing controls, the primary purpose of these bacterial culture samples was to enable unambiguous assignment of ASVs retrieved from the coral spat samples to the specific inoculum strains.

For Symbiodiniaceae counts, spat ($n = 12$ per treatment) were fixed in 400 μL of 3% paraformaldehyde (PFA) prepared in FSW. Following an overnight incubation at 4°C, the samples were washed once in 1× phosphate-buffered saline (PBS) and placed in a solution of 0.5 M disodium ethylenediaminetetraacetate (Na_2EDTA; pH 7.8) prepared in 1× PBS for decalcification (Fautin and Mariscal 1991). The samples were stored in the Na_2EDTA solution at 4°C for approximately 6 months until microscopy examination. Samples for fluorescence in situ hybridisation (FISH) ($n = 10$ per treatment) were fixed in 400 μL of freshly made, cold 4% PFA (made in 3× PBS) and incubated for 14–16 h at 4°C (Ainsworth et al. 2006). The samples were washed twice with cold 3× PBS and once in cold 1× PBS, with each wash lasting 10 min. After the final wash, the spat were stored in 500 μL of a 1:1 ethanol:1× PBS solution at −20°C (Ainsworth et al. 2006). Note that samples for protein extractions and Symbiodiniaceae counts were collected only on Day 13 due to the limited number of available spat. Moreover, corals treated with *Ruegeria* exhibited tissue regression and experienced mass mortality by the end of the experiment, preventing sample collection on Day 17 of spat exposed to this treatment.

2.4 | Coral Basal Disk Area

Individually settled *A. kenti* spat were photographed on Days 2, 13, and 17 using a Nikon D810 camera equipped with an AF-S Micro Nikkor 60 mm lens (Nikon, Japan) and mounted on a fixed-height stand. A total of 263 spat were tracked over time, with a minimum of 21 spat measured per treatment. The basal disk area of each spat was determined with FIJI ImageJ (V 2.9.0), with measurements conducted semi-automatically using the Outline, Threshold, Measurements and Analyse Particles tools in the base FIJI platform (Thomas and Gehrig 2020). For each individual spat, the percentage increase in basal disk area on Day 13 was calculated relative to their Day 2 measurement (Hughes and Connell 1987).

2.5 | Symbiodiniaceae Cell Counts

The number of Symbiodiniaceae cells in individual coral spat was determined by fluorescence microscopy of squashed, decalcified individuals using a protocol modified from Spencer Altice (2024). For each treatment, four intact decalcified spat sampled on Day 13 were individually mounted on separate microscope slides and gently squashed by placing a weight on top of the coverslip. Slides were viewed and photographed using a Leica DMI6000 B (Leica Microsystems, Germany) inverted microscope equipped with LasX software (Leica application suite 2.0.0.14332) under both brightfield and fluorescence microscopy. Fluorescence imaging was conducted using the camera-DFC310 FX (Leica Microsystems) and filter cube I3 (excitation filter; BP 450–490; dichromatic mirror = 510; suppression filter: LP 515) with a metal halide lamp. This setup allowed for differentiation between the autofluorescent coral tissue (green) and Symbiodiniaceae cells (red) (Figure S1). Symbiodiniaceae

cell counts were estimated using FIJI ImageJ v2.3.0 (Schindelin et al. 2012) with the Colocalization Object Counter plug-in (Lunde and Glover 2020). Manual validation and adjustments were performed using the counter feature in the base FIJI software. Where clusters of overlapping symbiont cells were present, the number of cells was estimated based on the size and colouration of the cluster. The area of each spat was manually outlined using the freehand tool, and the resulting measurements were used to calculate the average number of Symbiodiniaceae cells per mm^2.

2.6 | Total Protein and Phenoloxidase Assays

Protein extraction and phenoloxidase (PO) enzyme assays were adapted from previously published protocols (Palmer, Graham, and Baird 2012; Van De Water et al. 2015) with some modifications. Briefly, coral spat ($n = 3$ pools of 20 individuals), previously sampled and stored at −80°C, were transferred to PowerBead tubes (pre-filled with garnet beads, ϕ 0.1 mm) obtained from DNeasy UltraClean Microbial Kits (Qiagen, Germany). A volume of 500 µL of 1× PBS (50 mM, pH 7.8; P4417, Sigma-Aldrich, United States) was added to each tube, and the samples were incubated on ice for 5 min. Tissue lysis was carried out using three cycles of bead beating at 4 m/s for 20 s each, using a FastPrep-24 system (MP Biomedicals, United States), with 3 min cooling intervals on ice between cycles. The samples were then centrifuged at 12,000×*g* for 45 min at 4°C, and the supernatant was transferred to clean 1.5 mL tubes which were kept at 4°C for subsequent steps.

The total protein content of each coral lysate was determined using the DC Protein Assay kit (Bio-Rad, United States) following the manufacturer's micro-assay protocol with some modifications. Briefly, 20 µL of each sample was loaded in triplicate into a 96-well microtiter plate (Sarstedt, Germany). Subsequently, 10 µL of Reagent A and 80 µL of Reagent B were sequentially added to each well. Using a Cytation 3 plate reader (Agilent BioTek, United States), the plate was gently agitated with an orbital shake for 10 s to ensure thorough mixing in each well, and absorbance was measured at 750 nm. The obtained values were used to calculate the total protein concentration of the lysate based on a standard curve using bovine serum albumin (2 mg/mL; 5,000,206, Bio-Rad, United States) at triplicate points over the range of 0–200 µg/mL protein.

To measure total potential phenoloxidase (tpPO) and PO activities, 20 µL of each coral lysate and 40 µL of 1× PBS (50 mM, pH 7.8) were loaded in triplicate into 96-well microtiter plates. For tpPO activity, 25 µL of trypsin (0.1 mg/mL; T4799, Sigma-Aldrich, United States) was added to each well, while for PO activity, 25 µL of Milli-Q water (Merck Millipore, United States) was used instead. The plates were incubated at room temperature for 20 min, after which 30 µL of dopamine hydrochloride (10 mM; H8502, Sigma-Aldrich, United States) was added to each well. The plates were incubated for a further 15 min, then absorbance at 490 nm was measured every 5 min for 45 min, for a total of nine measurements. Enzymatic activity was calculated based on the change in absorbance over the linear portion of the reaction curve and normalised to each sample's total protein concentration.

2.7 | DNA Extraction and Amplification

A modified protocol for low-biomass samples (Høj et al. 2009) was employed for DNA extraction, incorporating mechanical homogenisation, physical and chemical lysis, and phenol–chloroform extraction. Briefly, 500 µL of lysis buffer (40 mM Na_2EDTA, 0.75 M sucrose, 50 mM Tris–HCl, pH 8.3) was added to each pooled sample. Homogenisation was performed using three rounds of bead beating at 4 m s^{-1} for 20 s each with a FastPrep-24 system (MP Biomedicals) and 30 mg of acid-washed glass beads (size 710–1180 µm, G1152, Sigma-Aldrich) per tube. The lysate was transferred to fresh tubes and incubated at 37°C for 1 h after the addition of lysozyme (400 µg mL^{-1} final concentration, L3790, Sigma-Aldrich). Sodium dodecyl sulphate (SDS; 4% final concentration) was then added, and the samples were incubated at 70°C for 10 min, followed by three freeze–thaw cycles (−80°C and +70°C). Once the samples reached room temperature, Proteinase K (625 µg mL^{-1} final concentration, 25530049, ThermoFisher Scientific, United States) was added, and samples were incubated at 65°C for 1 h. DNA was extracted by sequential addition of equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1, pH 8, 77617, Sigma-Aldrich) and chloroform:isoamyl alcohol (24:1, 25666, Sigma-Aldrich), with samples centrifuged for 10 min at 16,000×*g* after each addition. The aqueous phase was then precipitated with an equal volume of 100% isopropanol and 0.1 M sodium acetate (final concentration). DNA was pelleted by centrifugation at 4°C and 16,000×*g* for 30 min, washed with 70% ethanol, air-dried for 15 min and resuspended in 30 µL UltraPure nuclease-free water (10977015, ThermoFisher Scientific). After overnight resuspension at 4°C, DNA quantity and quality were assessed using a NanoDrop ND-2000 spectrophotometer (ThermoFisher Scientific).

The V5 and V6 variable regions of the bacterial 16S rRNA gene(s) were amplified using PCR primers 784 F [5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGGATTAGATACCCTGGTA-3′] and 1061 R [5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCRRCACGAGCT GACGAC-3′] (Andersson et al. 2008; Röthig et al. 2016). Illumina overhangs are indicated by the underlined sequences. PCR reactions were conducted in 30 µL volumes using the AmpliTaq Gold 360 Master Mix (439881, ThermoFisher Scientific) and 0.4 µM of each primer. The thermocycling conditions consisted of an initial denaturation step at 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 60 s, and extension at 72°C for 30 s. A final extension step was carried out at 72°C for 7 min. The resulting PCR products were sent to the Ramaciotti Centre for Genomics (UNSW, Sydney) for purification, library preparation, and paired-end sequencing on the Illumina MiSeq platform (v2, 2×250 bp).

2.8 | Processing of 16S rRNA Gene Sequences and Amplicon Analysis

The resulting partial 16S rRNA gene sequences were processed using QIIME 2 (version 2022.2; Bolyen et al. 2019). Primers and adapters were removed with the cutadapt plug-in using a 0.1 error rate (Martin 2011). Interactive quality plots were then inspected to assess read quality. The DADA2 plug-in (Callahan

et al. 2016) was used to filter low-quality reads (Q score < 30, with the forward and reverse reads truncated at positions 173 and 138, respectively), perform dereplication, merge paired-end reads and identify chimaeras. Amplicon sequence variants (ASVs) were identified based on exact matches (100% identity) (Callahan et al. 2017). Taxonomic assignment was conducted using a trained naïve Bayes classifier via the feature-classifier plug-in (Bokulich et al. 2018) with the V5–V6 region of the 16S rRNA gene and the SILVA 138 database (Quast et al. 2013). Multiple ASVs with 100% identity to our strains were returned for several of our candidates, suggesting these strains contain multiple copies of the 16S rRNA gene with slight differences (Větrovský and Baldrian 2013). The feature table, taxonomic counts and metadata were imported into R v 4.2.1 (R Core Team 2024) for further analysis.

Data processing and visualisation were conducted in R (R Core Team 2024) using RStudio v2024.12.0.467 (Posit team 2024) and the packages phyloseq (McMurdie and Holmes 2013), tidyverse (Wickham et al. 2019) and ggplot2 (Wickham 2016) Potential contaminant ASVs were identified and removed using the decontam package (Davis et al. 2018). Negative controls, including eight extraction blanks and three PCR blanks, were excluded from the dataset. Samples were rarefied to a sequencing depth of 18,600 reads, which was deemed sufficient based on the plateau observed in rarefaction curves before 20,000 reads (Figure S2; Hughes and Hellmann 2005; Weiss et al. 2017).

Alpha diversity indices were calculated in the phyloseq package (McMurdie and Holmes 2013). To assess significant differences in the Shannon diversity index (Lande 1996; Legendre and Legendre 2012) between treatments and the placebo control, a general linear mixed effects model (glmmTMB) with a Gaussian family distribution (Brooks et al. 2017) was applied. Post hoc pairwise contrasts were conducted using the emmeans package (Lenth 2024).

Beta diversity was assessed using Bray–Curtis dissimilarity matrices (Anderson et al. 2006). Differences in beta diversity were tested via permutational multivariate analysis of variance (PERMANOVA; Anderson 2001) using the adonis2 function in the vegan package (Oksanen et al. 2024). Dissimilarity matrices were visualised with Principal Coordinate Analysis (PCoA; Legendre and Legendre 2012), and homogeneity of group dispersions (PERMDISP; Anderson et al. 2006) was evaluated using the Multivariate Homogeneity of Groups Dispersions function betadisper in vegan (Oksanen et al. 2024). Pairwise comparisons were conducted using the function pairwise.adonis2 (Martinez 2020) and p values were corrected using the Benjamin and Hochberg correction for multiple testing (Benjamini and Hochberg 1995).

Drivers in microbial diversity among treatments were investigated using Sparse Partial Least Squares Discriminant Analysis (s)PLS-DA (Lê Cao et al. 2011) and principal component analysis (PCA) in the mixOmics package (Rohart et al. 2017). The (s)PLS-DA model incorporates the predictor matrix (bacterial taxonomic abundance data) and the response vector (treatment). This approach allowed for the identification of important microbial taxa that differentiated between treatments while accounting for the compositional nature of the microbiome data. The PCA provided a complementary visualisation of overall community structure and variation across treatments and time points, facilitating broader comparisons of microbial community composition.

Differences in the relative abundance of inoculum-specific ASVs between Day 13 and Day 17 were evaluated using a two-sided Wilcoxon rank-sum test (Wilcoxon 1945) on unpaired samples, implemented in the stats package (R Core Team 2024). For treatments containing multiple inoculum-specific ASVs, their relative abundances were summed within each sample prior to conducting the comparisons.

Co-occurrence network analysis was performed by combining samples from the final two time points (Day 13 and Day 17) within each treatment group, in order to increase sample size and improve the robustness of the resulting networks. Bacterial association networks were constructed at the ASV level, retaining only ASVs with a relative abundance greater than 0.1% within each treatment. Network inference was conducted using the SPRING method (Semi-Parametric Rank-based approach for INference in Graphical models; Yoon et al. 2019), as implemented in the NetCoMi package (Peschel et al. 2021). A regularisation path of 50 lambda values was specified, with the minimum lambda set to 1×10^{-3} times the maximum value. To further reduce noise, only ASVs present in at least eight out of 10 samples within a treatment were included. All other parameters were kept at their default settings.

2.9 | Fluorescence In Situ Hybridization Microscopy (FISH)

Coral spat were decalcified, embedded in paraffin wax and sectioned to a thickness of 4 μm (Wada et al. 2016) at the Melbourne Histology Platform (School of Biomedical Sciences, the University of Melbourne). Two to seven coral spat were prepared per treatment and time point (Table S3), with over 10 sections examined per individual. FISH was performed following the method described in Wada et al. (2016) with modifications. Tissue sections were dewaxed in xylene (2 × 3 min), dehydrated in 100% ethanol (3 × 5 min) and air-dried. Sections were then treated with 0.2 M HCl for 12 min, followed by a wash with 20 mM Tris–HCl (pH 8.0) for 10 min. A hydrophobic barrier was drawn around the sections with a hydrophobic pen, and 18 μL of hybridisation buffer (35% v/v formamide, 0.9 M NaCl, 20 mM Tris–HCl, 0.01% SDS) mixed with 2 μL of oligonucleotide probe (final concentration 5 ng μL^{-1}) was added to each section. Slides were incubated at 46°C for 3 h and washed for 10 min at 48°C in preheated wash buffer (0.07 M NaCl, 20 mM Tris–HCl, 0.01% SDS) contained in 50 mL polypropylene tubes. After washing, sections were soaked in ice-cold distilled water for 10 s to remove excess salts, air-dried and mounted using CitiFluor CFM-3 Antifadent Mountant (Electron Microscopy Sciences, United States). Prepared slides were stored at 4°C in a light-proof box and visualised within 24 h.

The universal bacterial probe EUB338 (5′-GCW GCC WCC CGT AGG WGT-3′) (Amann et al. 1990) labelled with Atto550 fluorophore (excitation: 553 nm, emission: 576 nm; biomers.net, Germany) was used to target all bacterial groups. The nonsense probe nonEUB (5′-ACA TCC TAC GGG AGG C-3′) (Wallner

et al. 1993) labelled with Atto647 (excitation: 645 nm, emission: 663 nm; biomers.net, Germany) served as a negative control. Additionally, the *Endozoicomonas*-specific probe Endozoi663 (5′-GGA AAT TCC ACA CTC CTC-3′) (Bayer et al. 2013) labelled with Atto550 was used to confirm the presence of *Endozoicomonas* bacteria in coral tissues where coral-associated microbial aggregates (CAMAs) were observed. Tissue sections without probes were also prepared to evaluate coral tissue autofluorescence.

Slides were examined using a Nikon AR1 inverted confocal laser scanning microscope (CLSM; Nikon) and the NIS-Elements software. The virtual band mode was used to collect emission spectra specific to each fluorophore. Atto550 was excited with a 561.5 nm laser line and detected within the 570–640 nm range, Atto647 was excited with a 638 nm laser line and detected within the 650–700 nm range, and coral autofluorescence was imaged using a 486.8 nm laser line with detection in the 500–560 nm range. Resulting nd2 files were processed in ImageJ, where brightness and contrast adjustments were applied linearly across entire images as needed. In parallel, histological sections stained with haematoxylin and eosin (H&E) were visualised using brightfield microscopy to identify coral tissue structures.

2.10 | Cross-Compatibility Assay

Screening for antibacterial activity was performed using agar well-diffusion assays, with the target strain embedded in the agar and candidates applied to pre-cut wells. All strains were initially tested for antibacterial activity against the putative coral pathogen *V. coralliilyticus* P1. Once the experiment was completed, candidates were checked against each other to assess cross-compatibility for inclusion in a potential consortium, except for *Ruegeria arenilitoris* 2-ATT-W2, which induced coral mortality. Overnight cultures of the probiotic candidates were prepared as described above. Minimal marine agar (MMA; composition: 0.3% casamino acids, 0.4% glucose and 1% bacteriological agar per 1 L FSW) was autoclaved and split between sterilised Schott bottles (100 mL per bottle) before cooling to 44°C in a water bath. For each bottle, 45 μL cold (4°C), filter-sterilised (0.22 μm) 20% glucose solution was added, followed by 1 mL of inoculum (overnight culture) immediately before pouring strain-specific plates in duplicates. Once all plates were solidified, wells were aseptically cut into the agar using pre-cut sterilised pipette tips (ϕ 5 mm) and an in-house vacuum system. Each well was inoculated with a different candidate strain (35 μL). The plates were incubated at 28°C and checked for inhibition zones after 24 and 48 h. The presence of inhibition zones surrounding the wells was measured and interpreted as an indicator of antagonistic activity against the strain embedded in the agar (Goulden et al. 2012). Clearing zones were classified into three categories: 1–10 mm (+), 11–20 mm (++), and > 20 mm (+++).

2.11 | Statistical Analyses

For all host response measurements, the assumptions of normality and homogeneity of variances were tested using the Shapiro–Wilk test (Shapiro and Wilk 1965) and Bartlett's test (Bartlett 1937), respectively. These assumptions were satisfied for enzyme activity data (measured as change in OD_{490}, mg protein^{-1} min^{-1}). Differences in enzyme activities between treatments and controls were investigated using analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test for post hoc pairwise comparisons (confidence level: 95%). To compare changes in basal disk area (growth) and the number of harboured Symbiodiniaceae, the non-parametric Kruskal–Wallis rank-sum test (Kruskal and Wallis 1952) was employed. Post hoc pairwise comparisons were performed using Dunn's test (Dunn 1964) with Bonferroni correction (Bonferroni 1936) applied to adjust for multiple comparisons.

3 | Results

3.1 | Shifts in the Microbiome After Inoculation

A total of 5,045,328 high-quality reads were obtained from 130 sequenced samples, including *A. kenti* spat, bacterial and Symbiodiniaceae cultures and negative controls. After removing ASVs affiliated with chloroplasts, mitochondria and contaminants (identified from negative controls), the dataset comprised 2485 ASVs.

Alpha diversity index values are presented in Table S4. In several instances, bacterial inoculation resulted in reduced Shannon diversity indices for treatments relative to the placebo control (Figure 2A; Table S5). Specifically, Shannon's index of diversity was significantly lower for the *Halomonas*, *Endozoicomonas* and *Roseivivax* treatments than the placebo control at both time points (Figure 2A; Table S5). For the *Thalassobius* treatment, the Shannon index was significantly lower for the treatment than the placebo control on Day 13, but by Day 17 the indices were comparable. Conversely, for the *V. coralliilyticus* treatment, there was no difference from the placebo control on Day 13, but by Day 17 the Shannon index was significantly lower for the treatment. Within-treatment comparisons over time revealed distinct temporal trends. The *Halomonas* treatment showed a significant increase in Shannon diversity between Days 13 and 17, while significant decreases were observed in the *Endozoicomonas*, *Pseudoalteromonas* and *V. coralliilyticus* treatments during the same period (Table S5).

The early microbiome of *A. kenti* shifted over a short time period, with coral spat samples collected on Days 2 and 4 clustering separately from those collected on Days 13 and 17, including the placebo controls (Figure S3). Bacterial inoculation had a clear impact on bacterial community composition (PERMANOVA, $p < 0.05$), with all treatments showing significant dissimilarity to the placebo control on both Days 13 and 17 (Figure 2B; Table S6). Sample dispersion (assessed using PERMDISP with 9999 permutations) also differed significantly between the placebo control and the *Halomonas* and *Roseivivax* treatments on Day 13, and the *Pseudovibrio* treatment on Day 17 (Table S6); however, the centroid of these treatments was clearly separated from that of the placebo control (Figure S4). PCoA plots further illustrated these differences.

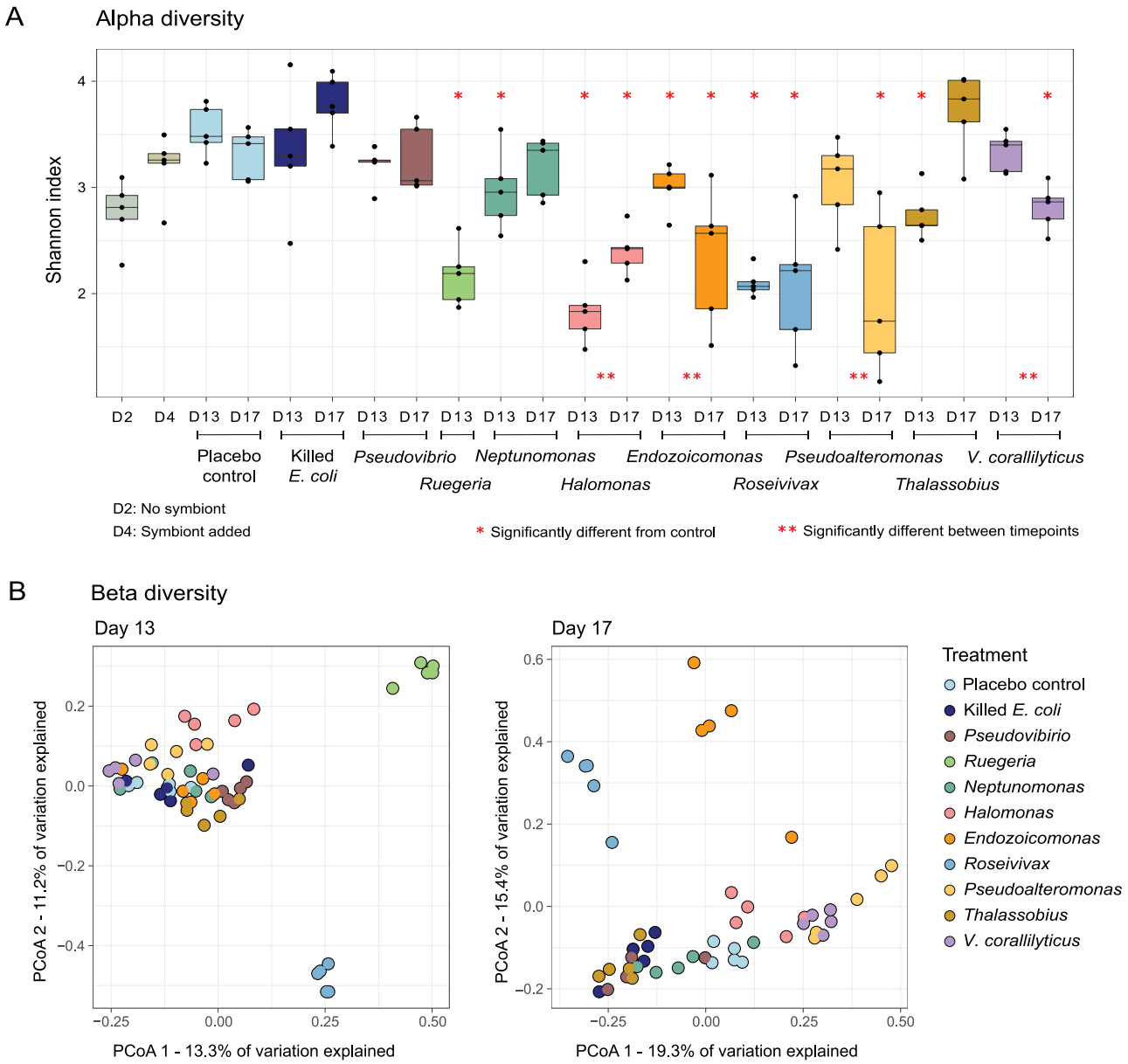

FIGURE 2 | Characterisation of the alpha and beta diversity of bacterial communities in *A. kenti* spat 1 and 5 days after the last inoculation with different bacterial strains (Days 13 and 17, respectively). (A) Box and whisker plots showing alpha diversity as measured by the Shannon index. Significant differences between treatments and the placebo control, and temporal changes within treatments are indicated with asterisks (see Table S5 for details). (B) Principal coordinate analysis plots showing beta diversity as measured by the Bray–Curtis dissimilarity index. Outcomes of relevant statistical tests (PERMANOVA, PERMDISP) are presented in Table S6.

On Day 13, samples from the *Ruegeria* and *Roseivivax* treatments formed distinct groups, separating from the placebo control and other treatments that clustered more closely together (Figure 2B). By Day 17, samples from the *Roseivivax* treatment continued to form a distinct cluster, and samples from the *Endozoicomonas* treatment clearly diverged from all other samples.

Shifts in beta diversity were generally influenced by ASVs associated with the inoculated bacterial strains (Table S7; Figure S5). On Day 13, differences between treatment groups and the placebo control were primarily driven by ASVs detected in the respective inoculum (Table S7; Figure S5), revealing a direct effect of the inoculum at this time point. This pattern persisted on Day 17 for most treatments, except for the *Pseudoalteromonas* and killed *E. coli* treatments, where beta diversity differences from the placebo control were no longer explained by inoculum-assigned ASVs (Table S7; Figure S6), suggesting an indirect restructuring of the coral microbiome in these treatments.

3.2 | Detection of Bacterial Inocula Over Time

For all treatments, the ASVs assigned to their respective inoculum were detected in all coral samples (Figure 3). The presence of multiple ASVs in the *E. coli*, *Neptunomonas*, *Endozoicomonas* and *V. coralliilyticus* inocula likely reflects the existence of multiple 16S

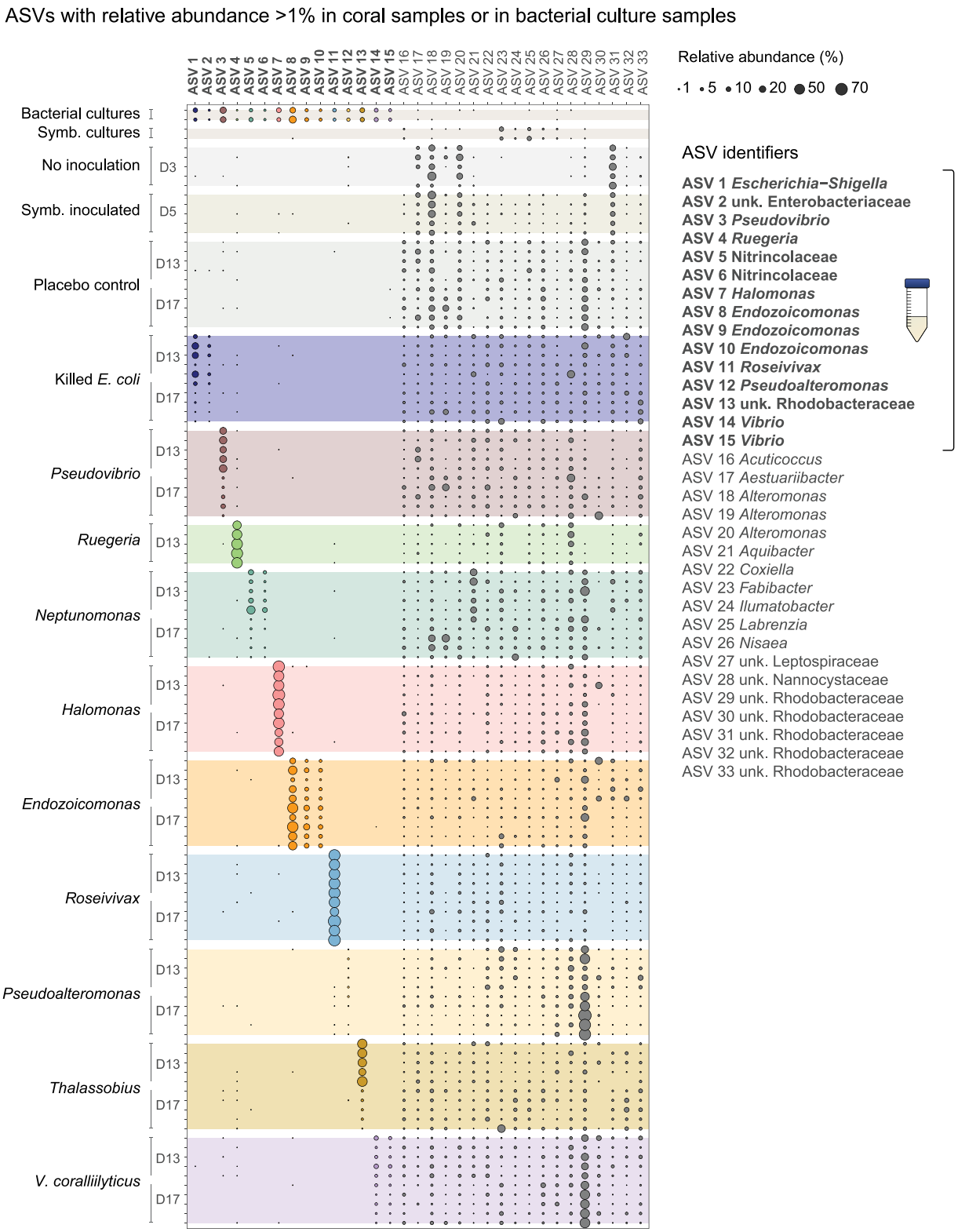

FIGURE 3 | ASVs selected for having a relative abundance above 1% in coral samples or above 1% in the bacterial culture samples (highlighted in bold at the top of the plot). The relative abundances of these ASVs are shown across bacterial and Symbiodiniaceae culture samples, as well as all *A. kenti* spat, which were sampled 1 and 5 days after the last inoculation with different bacterial strains (Days 13 and 17, respectively). The 'bacterial culture' samples are pooled samples of all inocula collected at two inoculation events. The 'Symb. culture' samples are from the Symbiodiniaceae culture used to inoculate coral spat on Day 2. Samples are grouped by treatment, as indicated on the *y*-axis and by the coloured shades. For the *Ruegeria* treatment, high spat mortality prevented sampling on Day 17 due to low remaining biomass. The ASV identifiers are reported in Table S7 with the corresponding ASV numbers. unk., 'unknown'.

rRNA gene copies typically found in most bacterial species (Pei et al. 2010) and within these genera, as indicated by the Ribosomal RNA Operon Copy Number Database (rrnDB; Stoddard et al. 2015). The relative sequence abundance of all ASVs varied depending on the treatment and time point (Figure 3). For instance, ASVs from the *Halomonas* and *Roseivivax* inocula were detected in the corresponding treatments at high relative abundance on Day 13, averaging 60.1% and 59.4%, respectively (Table 1). In contrast, ASVs assigned to the *Pseudoalteromonas* inoculum showed much lower prevalence, averaging just 0.59%. The relative sequence abundances of ASVs from other inocula fell within this range (Table 1). The *Roseivivax* and *Pseudoalteromonas* treatments maintained consistent relative abundances of inoculum-specific ASVs from Day 13 to 17 (Figure 3; Table 1). While the *Endozoicomonas* treatment exhibited a numerical increase in the average relative abundance of its inoculum-specific ASVs, rising from 30.2% on Day 13 to 57.0% by Day 17 (Figure 3), this increase was not statistically significant (Table 1). In contrast, the *Pseudovibrio*, *Neptunomonas*, *Thalassobius* and *V. coralliilyticus* treatments showed significant decreases in relative abundance of inoculum-specific ASVs over time, mirroring the pattern observed in the heat-killed *E. coli* treatment (Figure 3, Table 1). A statistically significant decline was also observed in the *Halomonas* treatment; however, the average relative abundance of its inoculum-specific ASVs remained high on Day 17 (42.1%) compared to these other treatments.

For all treatments, co-occurrence network analysis produced sparse and highly fragmented networks characterised by low clustering coefficients, poor connectivity and weak modularity with the presence of many singletons (Table S8). The limited sample size ($n = 10$ per treatment) could be a key factor contributing to sparse networks, as more samples per group are typically recommended for reliable network inference (Fabbrini et al. 2023). As such, the resulting co-occurrence patterns were deemed not to be robust or reliable enough to support further detailed interpretation or statistical comparison.

3.3 | Establishment of Coral-Associated Microbial Aggregates (CAMAs) in Spat Inoculated With *Endozoicomonas*

Tissue sections stained with H&E were examined to assess spat morphology and identify histological features (Figure 4A,B). The presence of CAMAs in the tissues of spat sampled from the *Endozoicomonas* treatment on both Days 13 and 17 was revealed by CLSM (Figure 4C–H). The CAMAs were round to oval, approximately 10 μm in size, and located within the epidermal mesenteries. Bacterial aggregates were initially detected with the universal EUB338 probe and later confirmed as *Endozoicomonas* using the Endozoi663 probe (Figure 4C,D; Table S3). At least 15 CAMAs were observed in a single spat sampled from this treatment on Day 13. Two spat sampled on Day 17 harboured at least two CAMAs each across examined sections. CAMAs were not detected in all spat analysed from the *Endozoicomonas* treatment, however, as there was no evidence of CAMAs found in one spat sampled on Day 13 and two spat sampled on Day 17. No CAMAs were observed in spat from any other treatment or the placebo control, and our approach failed to confidently detect individual bacterial cells in coral tissues.

3.4 | Host Responses to Bacterial Inoculations

As a proxy for immune response, the activity of the enzyme phenoloxidase of the melanin synthesis pathway was measured for spat sampled on Day 13, using dopamine as the substrate (Palmer, Bythell, and Willis 2012; Van De Water et al. 2015). No significant shifts in the average normalised activity of PO (Figure 5A) or tpPO (Figure 5B) were observed between treatments and controls. The *Ruegeria* treatment displayed the numerically highest PO activity, with a change in OD_{490} of 0.248 $(mg\,protein)^{-1}\,min^{-1}$, while the *Halomonas* treatment showed the numerically lowest activity, with an average change in OD_{490} of 0.105 $(mg\,protein)^{-1}\,min^{-1}$ (Figure 5A). For comparison, the placebo control had an average PO activity of OD_{490} of 0.166 $(mg\,protein)^{-1}\,min^{-1}$. Trends in tpPO activity mirrored those observed in PO activity but with slightly elevated values overall (Figure 5B).

Changes in basal disc area, a measure of spat growth, did not show statistically significant differences between treatments (Figure S7A). However, in the *Thalassobius* treatment, three spat exhibited substantial increases in basal disc area, ranging from 86% to 136%, compared to a maximum growth of 40% in the placebo control (Figure S7A). Tissue regression was observed in the *Ruegeria* treatment by Day 13, preventing accurate measurements of basal disc area. Finally, Symbiodiniaceae cell density exhibited high variability within all treatments, with no statistically significant differences detected (Figure S7B).

3.5 | Interactions Between Bacterial Cultures

In the agar diffusion assay (Table S9), *P. rubra* ATM06, *Neptunomonas* sp. 2-ATT-W3 and *P. denitrificans* 2-ATT-B1 exhibited antimicrobial activity after 24 h. *P. rubra* ATM06 inhibited all seven target strains, while *Neptunomonas* sp. 2-ATT-W3 and *P. denitrificans* 2-ATT-B1 inhibited four target strains each. *E. acroporae* 2-ATT-W5 and *T. mediterraneus* AT-O-15 allowed growth of all candidates in the first 24 h, but growth inhibition was evident for *R. lentus* 3-ATT-3 after 48 h. In contrast, *R. lentus* 3-ATT-3 and *H. smyrnensis* 2-ATT-W4 allowed all target strains to grow without inhibition (Table S9). *R. arenilitoris* 2-ATT-W2 inhibited growth of *V. coralliilyticus* P1 but was excluded from further cross-inhibition testing due to the high coral mortality in the *Ruegeria* treatment.

4 | Discussion

Probiotics, including those designed for coral, are commonly administered as consortia to maximise potential beneficial effects on host health and resilience (Puvanasundram et al. 2022). The assembly of effective consortia relies, however, on initial testing of candidates in isolation to determine their distinct roles in modulating the host microbiome and promoting host health. Moreover, understanding the propensity of individual candidates to establish a stable association with the host and/or contribute to long-term shifts in the microbial community is essential to evaluate the suitability of probiotic candidates (Thatcher et al. 2022; Li et al. 2023), with implications for application logistics and the development of targeted, efficient interventions that can be reliably implemented in restoration strategies.

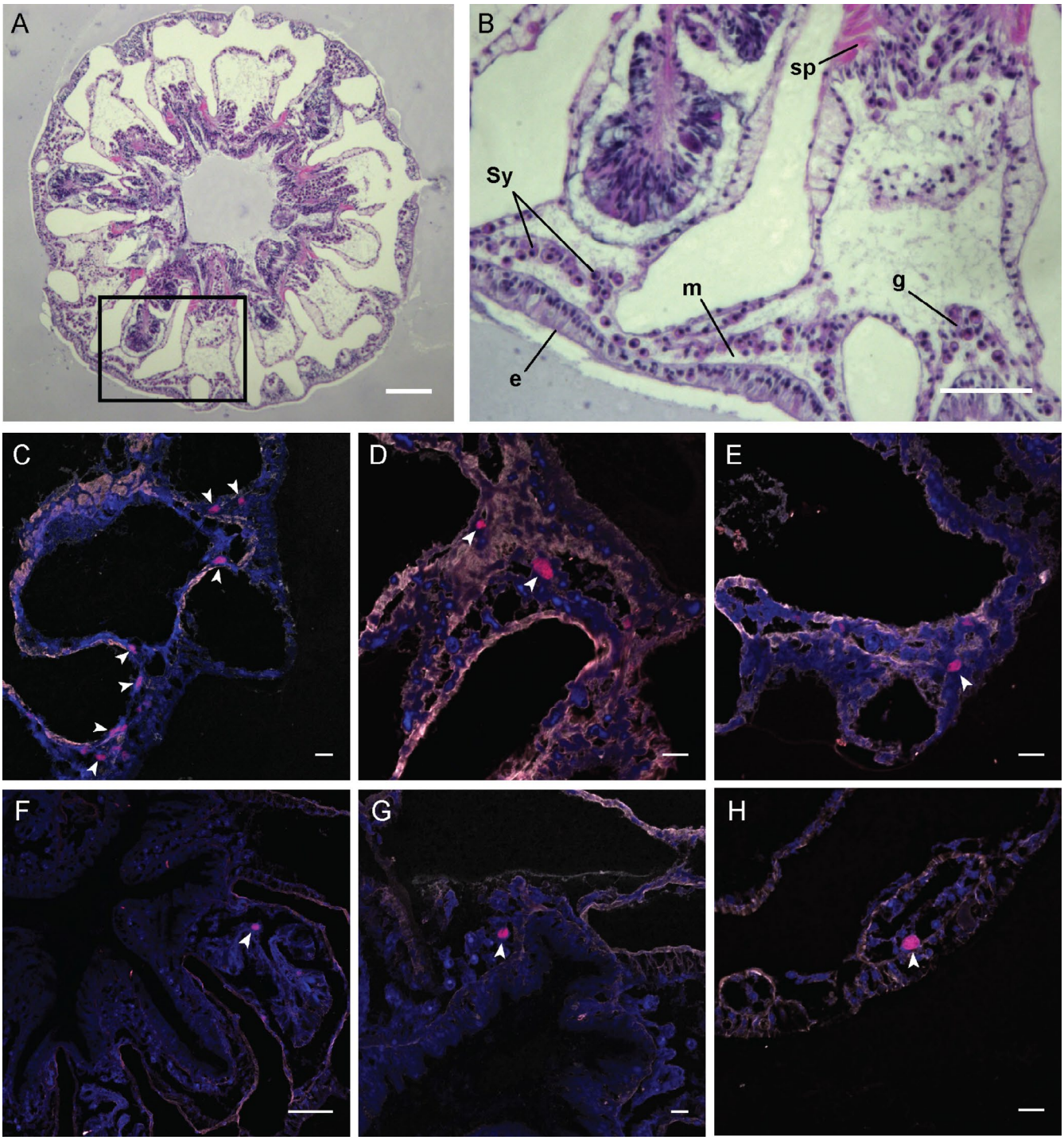

FIGURE 4 | Representative micrographs of histological sections of *A. kenti* spat (A and B) and detection of coral-associated microbial aggregates (CAMAs) by confocal microscopy following fluorescence in situ hybridization (FISH) analysis (C–H). (A) A horizontal cross-section of a 17-day old coral spat stained with haematoxylin and eosin (H&E), with the boxed zone enlarged in B. (B) Close-up of tissue section shown in (A); e, epidermis; g, gastrodermis; m, mesoglea; sp., spirocyte; Sy, Symbiodiniaceae cells. (C–H): location of CAMAs in a 13-day old (C–E) and a 17-day old (F–H) coral spat inoculated with *Endozoicomonas acroporae* 2-ATT-W5. Blue: coral tissue autofluorescence; red: EUB338 probe targeting all bacteria (C, F and H) or Endozoi663 probe targeting Endozoicomonadaceae (D, E and G); white: NonEUB probe showing unspecific binding (C–H). Scalebars: 10 μm (C–E, G–H), 50 μm (B and F), 100 μm (A).

In this study, we employed a highly controlled experimental system to investigate microbiome shifts in newly settled *A. kenti* spat following repeated inoculations with single probiotic candidates. The short study duration reflects its purpose as an initial screening tool to identify suitable probiotic candidates for further testing as part of a consortium over longer timeframes. Our results show that sustained bacterial applications significantly altered the bacterial community composition in coral spat over the brief 17-day experiment. Notably, several inoculum-associated ASVs persisted at high relative abundances 5 days

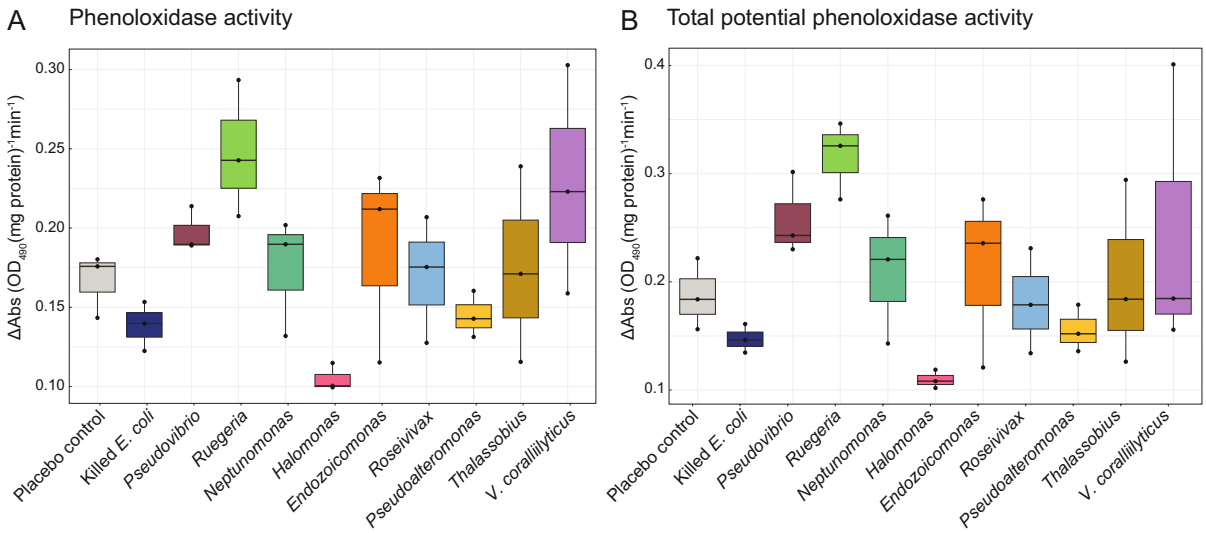

FIGURE 5 | Activity of phenoloxidase (A) and total potential phenoloxidase (B) in *A. kenti* spat 1 day after the last inoculation with different bacterial strains (Day 13). Horizontal bars indicate the median activity of three pooled samples per treatment and control, with each data point representing the average of three technical replicates. ΔAbs, difference in absorbance.

after the final inoculation. Furthermore, inoculation with *E. acroporae* 2-ATT-W5 led to the formation of CAMAs. While no significant effects on coral growth, Symbiodiniaceae density and phenoloxidase activity were observed during the experiment, treatment with *R. arenilitoris* 2-ATT-W2 resulted in tissue regression and high mortality. These findings highlight the potential for targeted microbiome manipulation during early coral life stages and emphasise the importance of individually screening probiotic candidates before combining them into consortia.

4.1 | Newly Settled Spat Were Susceptible to Microbiome Manipulation

Inoculation with bacteria triggered significant changes in the community composition of spat in all treatments at both time points (PERMANOVA, Table S6). These community shifts relative to the placebo control were primarily driven by ASVs assigned to the respective inocula (Figures S5 and S6). The relative abundance of the different inoculum-associated ASVs showed substantial variation, which is consistent with findings in previous studies on both adult (Rosado et al. 2019; Santoro et al. 2021) and juvenile corals (Damjanovic et al. 2019). Notably, these ASVs displayed contrasting temporal dynamics over the following 4 days (Figure 3, Table 1), further suggesting that the tested candidates varied in their ability to form stable associations with the coral host.

In the *Endozoicomonas* treatment, the relative abundance of inoculum-associated ASVs remained high (> 50%) 5 days after the final inoculation (Figure 3, Table 1), and FISH analysis demonstrated for the first time that inoculation with *Endozoicomonas* can lead to the formation of CAMAs in coral spat (see below). The genus *Endozoicomonas* has been reported as the dominant bacterial taxon in many acroporid corals, where it likely plays key roles in certain metabolic pathways such as dimethylsulfoniopropionate (DMSP) degradation, carbon cycling or vitamin B biosynthesis (Tandon et al. 2020; Pogoreutz et al. 2022; Pogoreutz and Ziegler 2024). As such, the *Endozoicomonas*–coral relationship is widely considered mutualistic, enhancing the holobiont metabolic efficiency and resilience. Negative correlations between the abundance of *Endozoicomonas* and coral stress or disease have also been observed (Meyer et al. 2014; Morrow et al. 2014; Pantos et al. 2015; McDevitt-Irwin et al. 2017), suggesting a protective role of *Endozoicomonas* for the coral holobiont against certain pathogens. However, the precise nature of this association remains debatable. In a recent review, Pogoreutz and Ziegler (2024) proposed that *Endozoicomonas* may span the symbiotic spectrum, ranging from mutualism and commensalism to opportunism and parasitism. Support for these non-mutualistic roles comes from genomic evidence and detrimental associations in other marine animal hosts (reviewed in Pogoreutz and Ziegler 2024). Hence, further studies are required to determine whether *Endozoicomonas* would be a suitable coral probiotic candidate.

The formation of a stable coral-bacterium association was also suggested in the *Roseivivax* treatment, where the relative abundance of inoculum-associated ASVs remained high (> 50%) 5 days post-inoculation. The genus *Roseivivax* belongs to the family Roseobacteraceae, previously known as the 'roseobacter clade' (Liang et al. 2021), which has been repeatedly found closely associated with spawning corals (Ceh et al. 2012, 2013) and within coral early life stages (Apprill et al. 2009, 2012). Many members of the family are implicated in DMSP cycling (Curson et al. 2017; Liang et al. 2021; Moran et al. 2003; Raina et al. 2010), including our inoculum strain *R. lentus* 3-ATT-3 (Kuek et al. 2022), and the production of bioactive compounds relevant for probiotic activities (D'Alvise et al. 2012; Bentzon-Tilia and Gram 2017). Specifically, a *Roseivivax* strain was found to induce coral larval settlement (Sharp et al. 2015), underscoring their potential importance in coral early life stages and development. The *Roseivivax* genus also includes many representatives capable of aerobic anoxygenic photosynthesis via bacteriochlorophyll and auxiliary pigments such as carotenoids (Suzuki et al. 1999; Chen et al. 2012; Jung et al. 2014),

including a strain isolated from the coral *Isopora palifera* (Chen et al. 2012). Most aerobic phototrophic bacteria have membranes that are highly enriched in carotenoids, which may play a role in photoprotection and the scavenging of singlet oxygen, free radicals or both (Yurkov 2006; Koblížek 2015). Further investigations are warranted to determine if the *R. lentus* 3-ATT-3 strain used in this study possesses these traits.

For the *Halomonas* treatment, the results were inconclusive. The inoculum ASVs maintained a high relative abundance (> 40% of ASVs) 5 days post-inoculation (Figure 3); however, there was a significant reduction in relative abundance (Table 1; from 60.1% to 42.1%) and a significant increase in the Shannon alpha diversity index (Figure 2A) as compared to the previous time point, raising questions about the ability of *H. smyrnensis* 2-ATT-W4 to successfully colonise *A. kenti* spat. While a previous study reported the detection of the genus *Halomonas* in *P. damicornis* before and after inoculation (Cardoso et al. 2024), we could not confidently visualise *Halomonas* cells in *A. kenti* spat. The genus *Halomonas* has been correlated with photosymbiosis in invertebrates (Bourne et al. 2013), and members carry genes involved in nitrogen cycling, vitamin B synthesis and possess other traits that may be beneficial to the coral holobiont (Rosado et al. 2019, 2023). While colonisation of the coral host was not confirmed here, further studies are warranted if similar beneficial traits can be confirmed for our *H. smyrnensis* 2-ATT-W4 strain.

Previous studies have shown that restructuring of bacterial communities after probiotic application can prompt coral transcriptional reprogramming and improved coral health outcomes, even when the probiotic itself is present at low levels or is undetectable (Santoro et al. 2021; Zhang et al. 2021; Li et al. 2023). With the exceptions outlined above (i.e., *Endozoicomonas*, *Roseivivax* and *Halomonas* treatments), all other inoculum-associated ASVs in our study were detected at relative abundances < 4% 5 days after inoculation (Table 1; Figure 3). These treatments could nevertheless provide potential host benefits if the bacterial community restructuring persists (Santoro et al. 2021), or if re-application of the probiotic candidate is logistically feasible (Thatcher et al. 2022). For example, substantial microbiome restructuring was observed in the *Pseudoalteromonas* treatment 5 days after the last inoculation, where unique ASVs emerged as key drivers of beta diversity shifts relative to the placebo control (Figure S6G). These ASVs were absent from the placebo control and other treatments, suggesting that *P. rubra* ATM06 may have indirectly facilitated microbiome restructuring over time, possibly via its antibacterial activity. Interestingly, Rosado et al. (2019) used a bacterial consortium comprising a *Halomonas*, a *Cobetia* and five *Pseudoalteromonas* strains to inoculate *P. damicornis* exposed to heat stress and pathogen challenge. While bleaching was partially mitigated in corals inoculated with the consortium, none of the *Pseudoalteromonas* strains were identified as bioindicators for the probiotic effect. The authors proposed that the consortium may have conferred indirect benefits by promoting microbiome restructuring. Members of the genus *Pseudoalteromonas* have also been shown to induce coral larval settlement and metamorphosis (reviewed in Peixoto et al. 2021; Turnlund et al. 2025). The potential of *Pseudoalteromonas* to influence microbiome assembly warrants further investigation, particularly during early coral ontogeny, when the microbiome is still developing and may be more susceptible to external modulation (Figure S3, Damjanovic, Menéndez, et al. 2020, Littman et al. 2009; Lema et al. 2014).

For the *Pseudovibrio, Neptunomonas*, *Thalassobius* and *V. coralliilyticus* treatments, the temporal dynamics of inoculum-specific ASVs were similar to those observed after the addition of heat-killed *E. coli*, suggesting that these bacteria may not have formed close associations with the coral host. In the latter treatment, ASVs assigned to *E. coli* showed a 10-fold reduction in average relative sequence abundance between one and 5 days after the final inoculation, indicating that the killed bacteria were likely consumed or phagocytised by the coral or its associated microbiota (Wild et al. 2004). Although killed bacterial cells can stimulate host immune responses, as reported in some postbiotic approaches (Taverniti and Guglielmetti 2011; Aguilar-Toalá et al. 2018), our immune response proxy assays revealed no activation of the melanin synthesis pathway in *A. kenti* spat following exposure to heat-killed *E. coli*. These results suggest that heat-killed *E. coli* serves as a suitable baseline for assessing the effects of other inocula on *A. kenti* spat, with minimal changes in the measured immune response proxy observed in this short-term study.

The detection of sequences from killed bacteria in coral samples highlights that next-generation sequencing cannot differentiate between viable and dead cells or extra-cellular DNA (Emerson et al. 2017), which may lead to erroneous conclusions regarding host–bacterial associations, particularly when assessing the fate of inoculated bacterial strains. To overcome this limitation, a combination of viability staining with propidium monoazide (PMA) (Nocker et al. 2009) and droplet digital PCR (ddPCR; Vandeputte et al. 2017; Gobert et al. 2018; Jian et al. 2020) has recently been applied to gain insight into absolute abundances of seawater microbiomes (Thomas et al. 2025) and several coral species (Dungan et al. 2023), and such approaches hold promise for future coral probiotic trials to provide unbiased insights into microbial community composition and host-bacterial interactions.

4.2 | Corals Treated With *Endozoicomonas* Formed CAMAs

The formation of CAMAs in the *Endozoicomonas* treatment only provides strong evidence for the establishment of symbiosis and replication of the introduced strain within coral tissues. The early life microbiome of *A. kenti* is primarily acquired through horizontal transmission (Damjanovic, Menéndez, et al. 2020), and while *Endozoicomonas* have been detected previously in coral early life stages through amplicon sequencing (Bernasconi et al. 2019; Damjanovic, Menéndez, et al. 2020), this is to the best of our knowledge the first report of CAMAs in early juveniles of *A. kenti* or other acroporid corals. While CAMAs in young acroporids remain undocumented, associations with *Endozoicomonas* are frequently recognised as key members of scleractinian coral microbiomes globally (Bayer et al. 2013; Neave et al. 2016). Several acroporid species, including *A. kenti* (Glasl et al. 2019; Damjanovic, Menéndez, et al. 2020), *A. digitifera* (Bernasconi et al. 2019), *A. humilis* (Bayer et al. 2013) and *A. loripes* (Damjanovic, Blackall, et al. 2020) often exhibit a predominance of *Endozoicomonas*. Additionally, *Endozoicomonas*

aggregates have been observed in adult corals of *Stylophora pistillata* (Bayer et al. 2013; Wada et al. 2022), *Pocillopora verrucosa* (Neave et al. 2016), *P. acuta* (Maire et al. 2023) and *A. loripes* (Gotze, Dungan, et al. 2025).

It remains unclear why *E. acroporae* 2-ATT-W5 and not the other strains used in this study formed CAMAs and persisted in coral tissues. Recent genomic analyses of coral-associated *Endozoicomonas* have identified eukaryotic-like genes that may facilitate host infection and suppress immune responses (Tandon et al. 2020; Ide et al. 2022; Hochart et al. 2023; Maire et al. 2023; Gotze, Tandon, et al. 2025). Further investigations are required to confirm which specific features enable *Endozoicomonas* to colonise coral tissues. To date, the only other bacterial taxa reported to form aggregates in corals are *Simkania* (Maire et al. 2023) and other chlamydia- or rickettsia-like bacteria (Work and Aeby 2014), groups known for their parasitic roles in animal hosts (Storz 1990). Ongoing comparative genome analyses aim to identify genetic features in the *E. acroporae* 2-ATT-W5 strain used in this study that could be implicated in its ability to infect coral tissues.

In addition to CAMAs, our study observed the retention and increased abundance of *Endozoicomonas*-associated ASVs after a 4-day recovery period without further inoculations. This is notable because stable associations with probiotics are uncommon in aquaculture systems, where repeated administration is often necessary for sustained benefits (Cruz et al. 2012). These findings suggest that the introduced *E. acroporae* 2-ATT-W5 strain may establish persistence and confer lasting benefits without continuous supplementation. Nevertheless, long-term monitoring of young corals treated with this strain is crucial to validate the durability and nature of this association.

4.3 | Host Responses to Bacterial Inoculations

Neither coral growth (basal disk area change) nor Symbiodiniaceae abundance counts varied significantly between individual probiotic treatments and control samples. Given that scleractinian corals are long-lived and slow-growing organisms, it is unsurprising that the basal disk area showed no significant response to probiotic treatment in this short-term trial. The high variability of Symbiodiniaceae counts observed within treatment groups aligns with findings from other studies on early life stage acroporid corals, which reported substantial within-sample variation in Symbiodiniaceae counts during the initial months following settlement (Quigley et al. 2020; Brunner et al. 2022). Future experiments extending over longer time periods may be necessary to determine if these probiotic treatments can influence coral growth rates or Symbiodiniaceae abundance. Additionally, more precise quantification of Symbiodiniaceae density in coral juveniles could be achieved in future studies by homogenising the decalcified coral tissue and counting individual symbiont cells using a haemocytometer (Quigley et al. 2023).

A. kenti spat inoculated with *R. arenilitoris* 2-ATT-W2 exhibited pronounced tissue loss and high mortality by Day 13. Although PO and tpPO activity levels were slightly elevated compared to the placebo control, the differences were not statistically significant, making it unclear whether an immune response was triggered. Further experiments with larger sample sizes, additional substrates targeting different steps of the melanin synthesis pathway (Brown et al. 2021), or gene expression analyses may be required to confirm these observations and determine whether *A. kenti* spat activate general defence mechanisms when exposed to this *Ruegeria* strain.

Despite the lack of conclusive evidence for immune activation, this in vivo trial highlights potential concerns with *R. arenilitoris* 2-ATT-W2 under the tested conditions and underscores the importance of individually screening probiotic candidates before incorporating them into consortia. In contrast to our findings, other *Ruegeria* strains have been associated with coral health benefits (Voolstra et al. 2024), with roles in carbon and sulphur cycling (Durham et al. 2015; Wirth et al. 2020; Sweet et al. 2021) and protection against pathogens such as *V. coralliilyticus* (Miura et al. 2019; Kitamura et al. 2021). Some strains have also been recommended for inclusion in coral probiotic consortia based on putatively beneficial traits (Doering, Tandon, et al. 2023; Dungan et al. 2021), and successfully applied in experimental probiotic trials (Xu et al. 2024).

Interestingly, inoculation with the known coral pathogen *V. coralliilyticus* P1 (Sussman et al. 2008) did not lead to any visible signs of disease or increased PO levels (Figure 5A) in *A. kenti* spat. This finding could be attributed to the reduced pathogenicity of the strain, potentially due to long-term storage and repeated subculturing under laboratory conditions (Austin 2017).

4.4 | Cross-Compatibility Assays as a Foundation for Probiotic Consortium Design

The advantages of multi-strain probiotics are well established, with mechanisms including increased biofilm formation (Puvanasundram et al. 2022), niche specialisation (Goulden et al. 2012) or other synergistic and additive effects (Timmerman et al. 2004; Lee et al. 2024). However, the design of a stable and effective consortium requires compatibility testing to avoid antagonistic interactions among the constituent strains (Prigigallo et al. 2023). Here, *H. smyrnensis* 2-ATT-W4 and *R. lentus* 3-ATT-3 showed no inhibitory effects on the growth of other probiotic candidates, suggesting their suitability for use in a multi-strain formulation. The *E. acroporae* 2-ATT-W5 strain showed no inhibitory activity after 24 h; however, after 48 h, this strain inhibited the growth of the *R. lentus* 3-ATT-3 strain. It is unclear whether this would present an issue if these strains were supplied as part of a consortium, with the possibility that CAMA formation by *Endozoicomonas* could ensure niche separation between these strains in the host.

Despite the antagonistic activity of the *P. rubra* ATM06 strain against all other candidates, the historical use of *Pseudoalteromonas* in coral probiotic applications (Rosado et al. 2019; Santoro et al. 2021; Moradi et al. 2023; Ushijima et al. 2023) and its potential niche-specific benefits within the coral mucus layer (Shnit-Orland and Kushmaro 2009; Sweet et al. 2021) mean that this strain still warrants further investigation. The strain may hold value in specific consortium configurations or in sequential application strategies, particularly

if paired with strains that tolerate its inhibitory effects. These results underscore the critical role of cross-compatibility assays in the development of multi-strain probiotic formulations and application strategies.

5 | Conclusion

This study screened eight native bacterial candidates as potential probiotics for *A. kenti* spat under controlled laboratory conditions, providing valuable insights into their suitability as probiotic treatments for coral restoration. While initial evidence from the literature and host association data suggested all tested candidates might confer benefits, in vivo performance varied significantly. We propose to exclude *R. arenilitoris* 2-ATT-W2 from further probiotic trials on *A. kenti* due to its association with high spat mortality. Conversely, *E. acroporae* 2-ATT-W5 demonstrated resilience by producing CAMAs and persisting within coral tissues post-inoculation, indicating its potential for establishing long-term symbiosis. Cultures of *R. lentus* 3-ATT-3 and *H. smyrnensis* 2-ATT-W4 maintained high sequence relative abundance in the coral microbiome beyond the inoculation period, conferring logistical advantages for practical applications. Although *P. rubra* ATM06 was not detected at high abundance in the coral microbiome, it induced structural shifts and may function as a transient probiotic in aquaculture, with reduced environmental risks after release.

Critically, none of these four candidates triggered adverse immune responses or impaired coral growth or symbiont densities. Therefore, we recommend these strains for inclusion in consortium-based probiotic approaches aimed at enhancing coral spat resilience to the anticipated challenges often observed in high-density aquaculture systems. By identifying and screening compatible probiotic strains, this study contributes to the development of multi-strain, species-specific probiotics aimed at enhancing coral health and resilience, particularly in aquaculture settings.

Author Contributions

Callaway Thatcher: conceptualisation, investigation, methodology, data curation, formal analysis, visualisation, writing – original draft preparation, writing – review and editing. **Katarina Damjanovic:** conceptualisation, investigation, resources, methodology, data curation, formal analysis, visualisation, validation, writing – original draft preparation, writing – review and editing. **Felicity Kuek:** investigation, resources, methodology, data curation, writing – review and editing. **Patrick W. Laffy:** data curation, formal analysis, supervision, visualisation, writing – review and editing. **David G. Bourne:** conceptualisation, funding acquisition, supervision, writing – review and editing. **Lone Høj:** conceptualisation, funding acquisition, investigation, methodology, project administration, supervision, writing – review and editing.

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Ethics Statement

All prevailing national and international regulations and conventions, as well as normal scientific ethical practices, have been respected.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in National Center for Biotechnology Information at https://www.ncbi.nlm.nih.gov/, reference number PRJNA1222579.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.