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ARTICLE

Bacterial controlled mitigation of dysbiosis in a seaweed disease

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Disease in the marine environment is predicted to increase with anthropogenic stressors and already affects major habitat-formers, such as corals and seaweeds. Solutions to address this issue are urgently needed. The seaweed *Delisea pulchra* is prone to a bleaching disease, which is caused by opportunistic pathogens and involves bacterial dysbiosis. Bacteria that can inhibit these pathogens and/or counteract dysbiosis are therefore hypothesised to reduce disease. This study aimed to identify such disease-protective bacteria and investigate their protective action. One strain, *Phaeobacter* sp. BS52, isolated from healthy *D. pulchra*, was antagonistic towards bleaching pathogens and significantly increased the proportion of healthy individuals when applied before the pathogen challenge (pathogen-only vs. BS52 + pathogen: 41–80%), and to a level similar to the control. However, no significant negative correlations between the relative abundances of pathogens and BS52 on *D. pulchra* were detected. Instead, inoculation of BS52 mitigated pathogen-induced changes in the epibacterial community. These observations suggest that the protective activity of BS52 was due to its ability to prevent dysbiosis, rather than direct pathogen inhibition. This study demonstrates the feasibility of manipulating bacterial communities in seaweeds to reduce disease and that mitigation of dysbiosis can have positive health outcomes.

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INTRODUCTION

Global climate change and elevated anthropogenic pressures have been correlated with an increase in diseases in marine systems, which are likely to become more frequent or severe in the future [1–3]. Seaweeds (macroalgae) are critical and dominant habitat formers in temperate coastal ecosystems, where they provide food and shelter for other marine organisms including invertebrates and fish [4, 5]. Disease outbreaks in habitat-forming seaweeds have caused massive declines in their population [3] and threatened species that rely on them [6, 7]. Recent reports have linked disease in marine hosts, including seaweeds, to opportunistic microbial pathogens [8, 9], and in some instances the causative agent(s) have been identified and virulence traits described [10–14]. However, up to now no effective strategies are available for disease control. Given that opportunistic pathogens may originate from the host-associated microbiota and/or the surrounding environment [15], effective disease management requires a holistic view considering the interplay between members of the host microbiota and the environment.

Delisea pulchra is one of the best-studied models for the interactions that occur between macroalgae, bacteria and the environment in the context of disease [16, 17]. This red macroalga suffers from a thallus bleaching disease, which is more prevalent in summer months due to an increase in seawater temperatures and a reduction of the seaweed's natural chemical defense, which is based on brominated furanones [18, 19]. Inoculation with specific bacteria (e.g. *Phaeobacter italicus* (formally, *Nautella italica*) R11 [18, 19], *Phaeobacter gallaeciensis* LSS9 [20], *Aquimarina* sp. AD1 and BL5, *Agarivorans* sp. BL7 and *Alteromonas* sp. BL110 [12]) can reproduce the disease phenotypes in chemically undefended individuals in the laboratory [12, 19, 20] or in the field [18]. Interestingly, chemically undefended seaweed individuals are frequently observed to be healthy in populations that generally suffer from bleaching disease [12], indicating that factors other than direct chemical defense can mediate disease resistance. A recent study following the succession of *D. pulchra*'s microbiome after a deliberate disturbance demonstrated that epiphytic microbial communities could recover to a pre-disturbed state within a relatively short time frame. However, preventing the establishment of specific bacterial colonists during the early succession compromised disease resistance of the host in situ [21]. These findings show that the composition or structure of the microbiota is an important factor in disease resistance and indicate that any imbalance, or dysbiosis, can contribute to disease development. In fact, microbial communities of bleached *D. pulchra* have been found multiple times to be distinct from those of healthy individuals in the field [18, 22–24] and in vitro inoculation with specific pathogens appear to cause a destabilisation of the seaweed's bacterial community structure [12].

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Controlled microbiota manipulation is increasingly recognised as a tangible practice to improve host health and performance, in particular in the area of human gut probiotics [25, 26] and in terrestrial crop production, e.g. plant growth-promoting microorganisms [27, 28]. The use of probiotics has also been extended to marine animals, like shrimp [29, 30], fish [31, 32] and more recently corals [33, 34]. Despite previous studies showing the disease-protective potential of bacteria associated with red seaweeds *Gracilaria conferta* [35] and *Agarophyton vermiculophyllum* [13], probiotics or microbiota manipulation is still understudied for the control of macroalgal diseases and mechanistic aspects are unknown.

Here, we hypothesise that manipulation of bacterial epibionts will result in the prevention or mitigation of the bacterial-induced bleaching disease of *D. pulchra*. To test this hypothesis, we determined the antagonistic activities against known *D. pulchra* pathogens for over 400 strains of epiphytic bacteria. Antagonistic bacteria were then assessed for their ability to prevent disease in vivo. We further compared the bacterial communities associated with *D. pulchra* that were inoculated with either a pathogen, a protective bacterium, a non-protective bacterium, or combinations thereof, to analyse the community shifts underpinning disease protection.

MATERIALS AND METHODS

Collection and screening for pathogen-antagonistic bacteria

Healthy *D. pulchra* individuals were collected in February 2018 off the coast of Cronulla Beach (34°03′23″S, 151°09′26″E), Sydney, from a depth of 3–4 m (permit: P13/0007-2.0 & OUT18/2054; New South Wales Department of Primary Industries). Samples were transported to the laboratory and rinsed individually in 0.22 µm filtered and autoclaved bromide-deficient artificial seawater (Br-ASW) [36] to remove loosely attached epiphytic bacteria. Br-ASW was used in all experiments to reduce the seaweed's bromide-furanone dependent chemical defense [36]. The thalli were swabbed using sterile cotton swabs, which were then transferred to Br-ASW and vortexed vigorously. Each suspension was serially diluted and spread onto Difco Marine Broth 2216 agar (MA) plates. After 6 days of incubation at 25 °C, bacterial colonies were isolated. Bacterial isolates were stored at −80 °C in 30% glycerol.

A diffusion-based agar plate bioassay [37] was used to assess the antagonistic activity of the bacteria isolated from healthy *D. pulchra* and bacteria previously isolated from marine surfaces (Table S1), against six known bleaching pathogens: *Phaeobacter italicus* R11 [19], *Phaeobacter gallaeciensis* LSS9 [20], *Aquimarina* sp. AD1 and BL5, *Agarivorans* sp. BL7 and *Alteromonas* sp. BL110 [12]. All bacteria were obtained from the culture collection of the Centre for Marine Science and Innovation (CMSI, UNSW Sydney). Stationary phase cultures of the pathogen were spread onto MA plates and air-dried for 1 min at room temperature. Discs containing 12 µL of test strains (grown overnight at 25 °C and 180 rpm) were placed onto the plate, incubated at 25 °C for 48 h and zones of growth inhibition were measured (an antagonistic effect was indicated by the zone of growth inhibition with a diameter ≥ 7 mm). For each test strain vs. pathogen pair, six discs (containing independent cultures of the test strain) were put on six different MA plates (spread with the pathogen) to estimate the variation associated with this method.

16S rRNA gene sequencing and analysis

PCR amplification of the 16S rRNA gene of pathogen-antagonistic bacteria were performed using primers 27 F/1492 R as described previously [38]. The PCR products were sequenced at the Ramaciotti Centre for Genomics, UNSW, Australia (for detailed procedure see Supplementary information). Forward and reverse reads were assembled for each bacterium and compared against the National Centre for Biotechnology Information (NCBI) NT database (July 2018) through the BLAST search [39]. The closest relatives were acquired, and all sequences were subject to phylogenetic analyses following the protocol of Hall, (2013) [40]. Briefly, sequences were aligned using the MUSCLE algorithm [41] in the integrated program Molecular Evolutionary Genetics Analysis (MEGA) X [42]. A phylogenetic tree was constructed using the Maximum Likelihood method [43] with 1000 bootstraps.

D. pulchra infection assay

Bacteria were tested in vivo for their ability to mitigate the bleaching disease using a previously developed method [12]. Briefly, healthy *D. pulchra* juveniles (ca. 6 cm in length, having dark-red thallus without any signs of disease or fouling) were collected from Bare Island (33°59′54″S, 151°14′59″E), Sydney, at 3–4 m depths, from April 2018 to September 2019, transported to the laboratory and cleaned following the procedure described above. The cleaned thalli were transferred individually into sterile 25 cm^2 Coring cell flasks (Merck, Germany) with vented caps. Each flask was filled with 50 mL of Br-ASW. Flasks were routinely maintained at 25 °C (60 rpm) and illuminated in a 14:10 h light-dark regime. Samples were maintained overnight under these conditions to acclimatise, and any dead, fading samples were excluded for the following inoculation experiments.

Bacteria that were from antibiotics producing taxa and exhibited in vitro antagonistic effects against multiple pathogens were chosen as representatives to screen for in vivo protective effects. For in vivo experiments, *Aquimarina* sp. AD1 was chosen as the model pathogen based on prior studies that indicate it is the most virulent [12, 44]. On day 1 healthy juveniles were randomly assigned to one of four groups: (i) no bacterial inoculation (control), (ii) inoculation with the pathogen *Aquimarina* sp. AD1, (iii) inoculation with a test bacterium, either *Phaeobacter* sp. BS52, *Phaeobacter* sp. BS23, *Phaeobacter* sp. BS34, *Vibrio* sp. BL95 or *Pseudoalteromonas* sp. PB2-1; and (iv) co-inoculation with each test bacterium and *Aquimarina* sp. AD1. Bacteria used in infection assay were cultured in Marine Broth 2216 (Difco) at 25 °C to reach log-phase growth. One millilitre of bacteria cells (concentration: 10^7 cfu·mL^{-1}) suspended in Br-ASW were prepared following the procedures described in [12] to inoculate *D. pulchra*. Test bacteria were inoculated at the start of the experiment with pathogen challenges occurring on day 2 and 4. The seawater was replaced with fresh Br-ASW before inoculations. On day 5, the juveniles were visually inspected for bleaching or discoloration of algal thalli. For each test bacterium a total of three independent experiments were conducted with at least three biological replicates per treatment (see Table S2 for details).

To determine the effect of treatment on disease incidence, the data were fitted to a Generalised Linear Mixed-effect Model (GLMM) assuming a binomial distribution using the *glmer* function in the lme4 R package [45]. 'Experiment' was included as a random effect in the model. The *glht* function in the multcomp R package was used to further assess the difference between treatments [46].

Post-infection analysis of *D. pulchra* bacterial community

Post-infection *D. pulchra* samples were rinsed with 15 mL 1 × PBS (Phosphate-Buffered Saline) buffer twice to remove the loosely attached bacteria from their surfaces. Total bacterial community DNA was extracted from algal samples using the DNeasy Powersoil Kit (Qiagen Australia, Chadstone, VIC, Australia) following the manufacturer's instructions. The primers with Illumina overhang adapter sequences, 341 F: 5′-tcgtcggcagc gtcagatgtgtataagagacagCCTACGGGNGGCWGCAG-3′ and 785 R: 5′-gtctcgtg ggctcggagatgtgtataagagacagGACTACHVGGGTATCTAATCC-3′ were used to amplify the V3-V4 regions of the 16S rRNA gene [47]. The reaction mixture (50 µL total volume per sample) consisted of Econo Taq PLUS GREEN 2× Master Mix (Astral Scientific, Gymea, NSW, Australia) (25 µL), Ambion nuclease-free water (Thermo Fisher Scientific, Australia) (15 µL), the primer pair 341 F and 785 R (2.5 µL of each; 10 µM) and DNA template (5 µL). The PCR program consisted of an initial denaturation at 94 °C (2 min), followed by 30 cycles of denaturation at 94 °C (30 s), annealing at 55 °C (30 s) and extension at 72 °C (40 s), and a final extension of 72 °C (7 min). PCR products were then quantified using gel electrophoresis. Addition of multiplexing indices and Illumina sequencing adapters, library preparation and paired-end sequencing (2 × 300 bp) of the resulting 16S rRNA gene amplicons was performed at the Ramaciotti Centre for Genomics, UNSW on a MiSeq platform (Illumina) as per the MiSeq System User Guide (Illumina 2013).

Raw sequencing reads were processed using TRIMMOMATIC version 0.36 [48] and USEARCH version 11.0.667 [49] following the procedure described in [50]. Briefly, sequences shorter than 100 bp were removed and the ends with a quality below 20 in a sliding window of 4 bp were trimmed. Contigs were made and the sequences with ambiguous or error bases, or with a length below 250 bp or exceeding 550 bp were removed. Filtered sequences were denoised and clustered into zero-radius operational taxonomic units (zOTUs) [51] using the UNOISE algorithm implemented in USEARCH. Chimeric sequences were removed with UCHIME [52] *de novo* during zOTU clustering and subsequently with a reference-based comparison against the GTDB v89 database (https://gtdb.ecogenomic.org/). zOTUs were taxonomically

classified using a Bayesian Last Common Ancestor algorithm (BLCA) [53] against the GTDB v89 database. All non-bacterial zOTUs and singletons were removed. Finally, the processed sequences were mapped onto zOTU sequences to calculate the count distribution of each zOTU in every sample. Only zOTUs occurring in more than two samples were considered for further statistical analyses. The sequencing depth was visualised with rarefaction curves and the sampling efficiency was estimated with Good's coverage indices using the R package vegan [54]. To account for the uneven sequencing depth, count data were subsampled to the lowest reads observed among samples (48 304) using USEARCH [49], and all further analyses were based on the subsampled data.

To investigate the presence of the inoculated bacteria (i.e. *Aquimarina* sp. AD1, *Phaeobacter* sp. BS52 and *Phaeobacter* sp. BS23) in our samples, the 16S rRNA gene sequences of the bacterial inocula were searched against the representative sequences of zOTUs using BLAST 2.2.30+ [39]. zOTU sequences with 100% identity (across the full V3–V4 region of the 16S rRNA gene that was sequenced) were considered as being derived from the inoculum. The relative abundances of each zOTU in samples treated or not treated with its corresponding inoculum were compared using a Generalised Linear Model (GLM) assuming a negative-binomial distribution, where 'Experiment' was included first in the model to account for potential variation among experimental trials, and the *p* values were calculated via 1000 parametric bootstraps [55].

Spearman's rank correlation tests were performed to assess the potential effect of *Phaeobacter* sp. BS52 and *Phaeobacter* sp. BS23 on the relative abundance of *Aquimarina* sp. AD1. Subsequent bacterial community analyses were performed after removal of sequences corresponding to the inocula. Alpha diversity indices, i.e. Shannon_e (the Shannon index logs to base e) for zOTU diversity and Chao1 for richness, were determined using USEARCH version 11.0.667 [49]. Analysis of variance (ANOVA) was performed to test the significance of the effect of 'treatment' on diversity indices. 'Experiment' was included as a factor first in the model to account for potential variation among experimental trials.

The R package vegan [54] was used for distance-based community analyses. To analyse the community structure, Bray–Curtis dissimilarity coefficients were calculated for the square-root transformed relative abundance data. The effects of 'treatment' on the community structure were tested by permutational multivariate analysis of variance (PERMANOVA) and pairwise comparisons with 9999 random permutations within each experiment (strata), using the R package pairwise.adonis2 [56]. The assumption of homogeneity of multivariate dispersion was tested using the *betadisper* function in the R package vegan. To identify bacterial taxa that contributed to the community differences, zOTUs were aggregated to different taxonomic levels (i.e. phylum to species). The relative abundances of different taxa (with mean relative abundance: ≥1%) were compared among treatments and experiments using a multivariate Generalised Linear Model (mGLM) assuming a negative-binomial distributionand *p* values calculated via 1000 parametric bootstraps [55]. 'Experiment' was included first in the model to account for potential variation among experimental trials. Where significant treatment effects were detected, the argument 'pairwise.comp' in the function *anova* of R package stats was used to assess the difference between treatments. Significance was determined with alpha = 0.05. All analyses and statistics were performed in R version 3.6.3 [57].

RESULTS

Identification of bacteria with antagonistic activities against pathogens

A total of 260 bacterial strains isolated from healthy *D. pulchra* and 174 strains obtained from an in-house bacterial culture collection (Table S1), were tested for antagonistic activities towards known bleaching pathogens. Of these 7.1% (31 different strains) inhibited the growth of the pathogens *Phaeobacter gallaeciensis* LSS9, *Aquimarina* sp. AD1 and/or BL5, *Agarivorans* sp. BL7 and/or *Alteromonas* sp. BL110 (Fig. 1).

In total 139 of the 434 tested bacteria have been classified (to a genus or lower taxonomic level, according to previous records in Table S1 and the 16S rRNA gene sequencing conducted in this study), and these 139 strains represented 27 genera (Table S3). The antagonistic bacteria belonged to seven genera, including 19 *Vibrio*, six *Phaeobacter*, two *Microbulbifer*, one *Bacillus*, one *Photobacterium*, one *Pseudoalteromonas* and one *Pseudomonas* (Fig. 2, Tables S1 and S4). Six *Vibrio* and four *Phaeobacter* bacterial strains displayed growth inhibition towards three of the pathogens (the two *Aquimarina* spp. and *Phaeobacter gallaeciensis* LSS9 or *Agarivorans* sp. BL7, see Fig. 1). None of the tested bacteria inhibited all six pathogens.

Identification of bacteria that protect *D. pulchra* from bleaching disease

Five bacterial strains (namely, *Phaeobacter* spp. BS23; BS34 and BS52, *Vibrio* sp. BL95 and *Pseudoalteromonas* sp. PB2-1) with potential to antagonise multiple opportunistic pathogens (as determined by both in vitro tests and their affiliations to antibiotics producing taxa) were selected for further in vivo testing of their ability to protect *D. pulchra* from disease. To assess if the inoculation of *D. pulchra* with any of the single strains (i.e. *Aquimarina* sp. AD1, *Phaeobacter* spp. BS23; BS34 and BS52, *Vibrio* sp. BL95 and *Pseudoalteromonas* sp. PB2-1) had an impact on the likelihood of disease, the proportions of healthy individuals after the experiment were compared to an uninoculated control. As expected, treatment with the pathogen *Aquimarina* sp. AD1 alone significantly reduced the proportion of healthy individuals (post-hoc tests on binomial GLMM, $Df = 114$, $p_{adjusted} < 0.001$) compared to the uninoculated controls. However, there was no statistical support for a beneficial or detrimental effect of the candidate protective bacteria on *D. pulchra* health in the absence of pathogen challenge (Fig. 3, Table S5).

Next, to determine whether any of the candidate protective bacteria could reduce the pathogen-induced bleaching, we compared the test strain+pathogen treatments with the pathogen challenge treatment (*Aquimarina* sp. AD1-only). Co-inoculations with *Phaeobacter* sp. BS52 or *Pseudoalteromonas* sp. PB2-1 significantly increased the proportion of healthy *D. pulchra* samples compared to the AD1-only treatment (post-hoc tests on binomial GLMM, $Df = 76$, $p_{adjusted} = 0.0486$; $Df = 81$, $p_{adjusted} = 0.0047$, respectively; Fig. 3, Table S5). There was no support for a statistically significant impact on disease protection for the other strains tested (i.e. BS23, BS34 and BL95).

Treatment with disease-protective bacteria mitigates pathogen-induced microbiome shifts in *D. pulchra*

We used a 16S rRNA gene-based community analysis to determine the relative abundance of selected disease-protective bacteria and to assess the effect of these strains on the overall bacterial community associated with *D. pulchra*. We chose to focus this analysis on the protective strain *Phaeobacter* sp. BS52, and a closely related strain *Phaeobacter* sp. BS23 (Fig. 2), for which there was no evidence of protection (Fig. 3). After quality filtering, a total of 5,681,299 sequences were generated for 52 seaweed samples (Table S6), which clustered into 1 488 zOTUs. Rarefaction curve and Good's coverage (>99.8%) indicated that the majority of the bacterial communities was recovered by the surveying effort (Table S7, Fig. S1).

The sequences of three zOTUs (namely, zOTU258, zOTU2 and zOTU5) matched perfectly and uniquely to the 16S rRNA gene of the inoculated bacteria (AD1, BS52 and BS23, respectively) and hence were assigned to these strains. This is further supported by the observation that a significant increase in the relative abundance of zOTU258, zOTU2 or zOTU5 occurred in samples treated with AD1, BS52 or BS23, respectively, compared to untreated samples (negative-binomial GLM; $Deviance = 4.7$, $p = 0.034$; $Deviance = 155.8$, $p = 0.001$; $Deviance = 132.1$, $p = 0.001$, respectively; Fig. S2).

To test the hypothesis that there would be antagonistic interaction in vivo between the disease protective bacterium and the pathogen, we performed Spearman's rank correlation analyses of their relative abundances. We did not find statistical support for a negative correlation between the relative abundance of *Aquimarina* sp. AD1 and *Phaeobacter* sp. BS52 (Spearman's rank correlation; $rho = 0.08$, $p > 0.05$).

We next tested the hypothesis that the addition of a protective bacterium influenced the microbiota associated with *D. pulchra*,

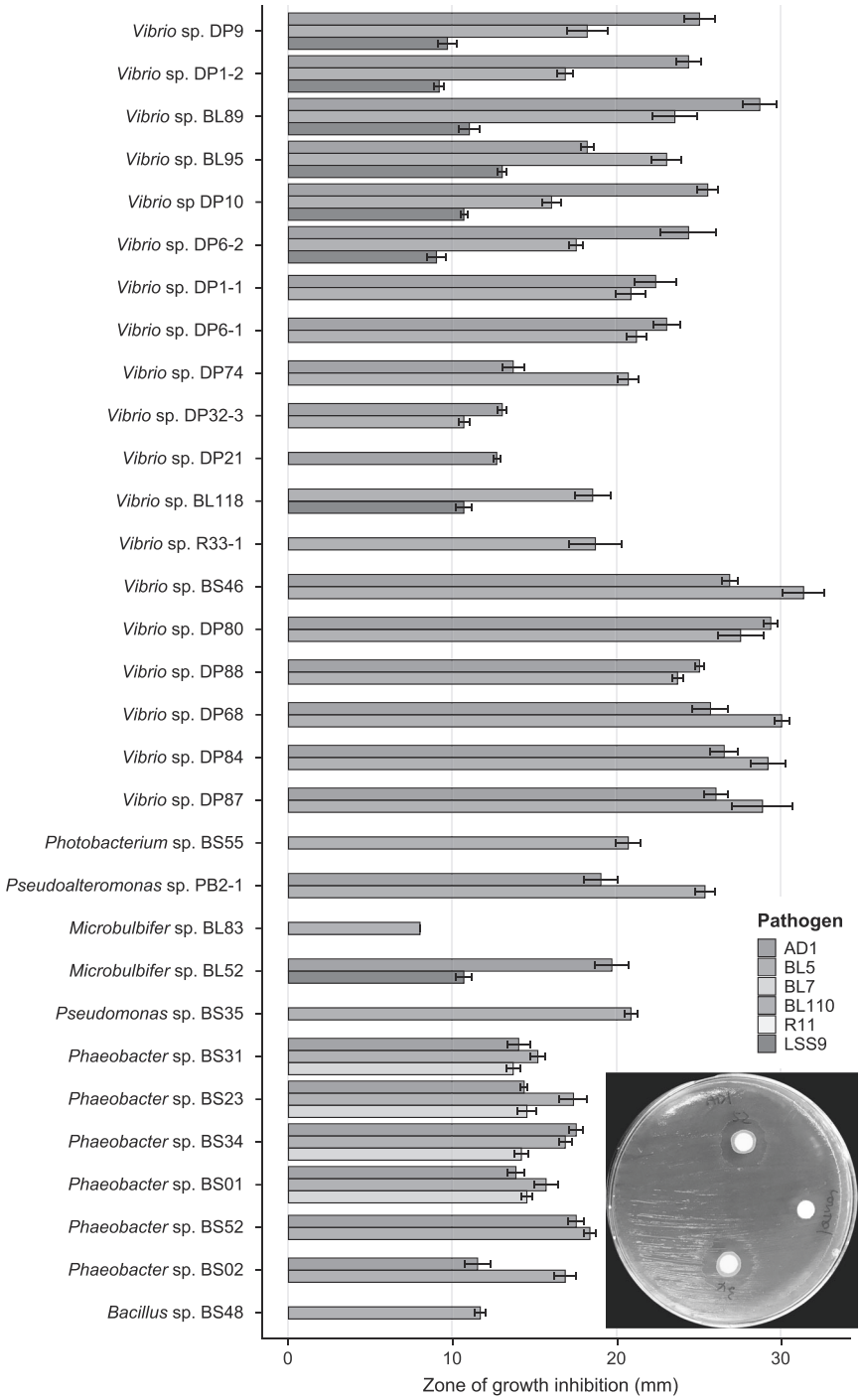

Fig. 1 Antagonistic activities of disease-protective candidates against known *Delisea pulchra* bleaching pathogens, *Phaeobacter italicus* R11, *Phaeobacter gallaeciensis* LSS9, *Aquimarina* sp. AD1 and BL5, *Agarivorans* sp. BL7 and *Alteromonas* sp. BL110. The ×-axis shows the zone of growth inhibition (mean diameter ± standard error in mm, based on $n = 6$ biological replicates). The *y* axis shows the taxonomic affiliation and identification number (ID) of test bacteria. An example plate viewed under natural light shows typical growth inhibition (inset plot at the bottom-right corner).

which in turn may prevent the bleaching disease. While there was not statistical support for difference in the alpha diversity of the bacterial communities associated with *D. pulchra* between the treatments (Table S8), an overall effect of treatments (PERMANOVA, *pseudo*-$F_{5,44} = 1.487$, $p = 0.002$, Table S9) was observed on the bacterial community structure based on Bray-Curtis dissimilarities (square-root transformed data). While pairwise comparisons showed no statistical support that the bacterial communities of *D. pulchra* co-inoculated with the protective *Phaeobacter* sp. BS52 and *Aquimarina* sp. AD1 differed from the control (i.e. no bacterial inoculation) ($p_{adjusted} = 0.089$), they were significantly different from those treated with AD1 alone ($p_{adjusted} = 0.001$) (Table S9). In contrast, we did not find statistical support for differences between the bacterial communities of *D. pulchra* co-inoculated with the non-protective *Phaeobacter* sp. BS23 and *Aquimarina* sp. AD1 and the AD1-alone treatment (Table S9). To further identify bacterial taxa that contributed to the

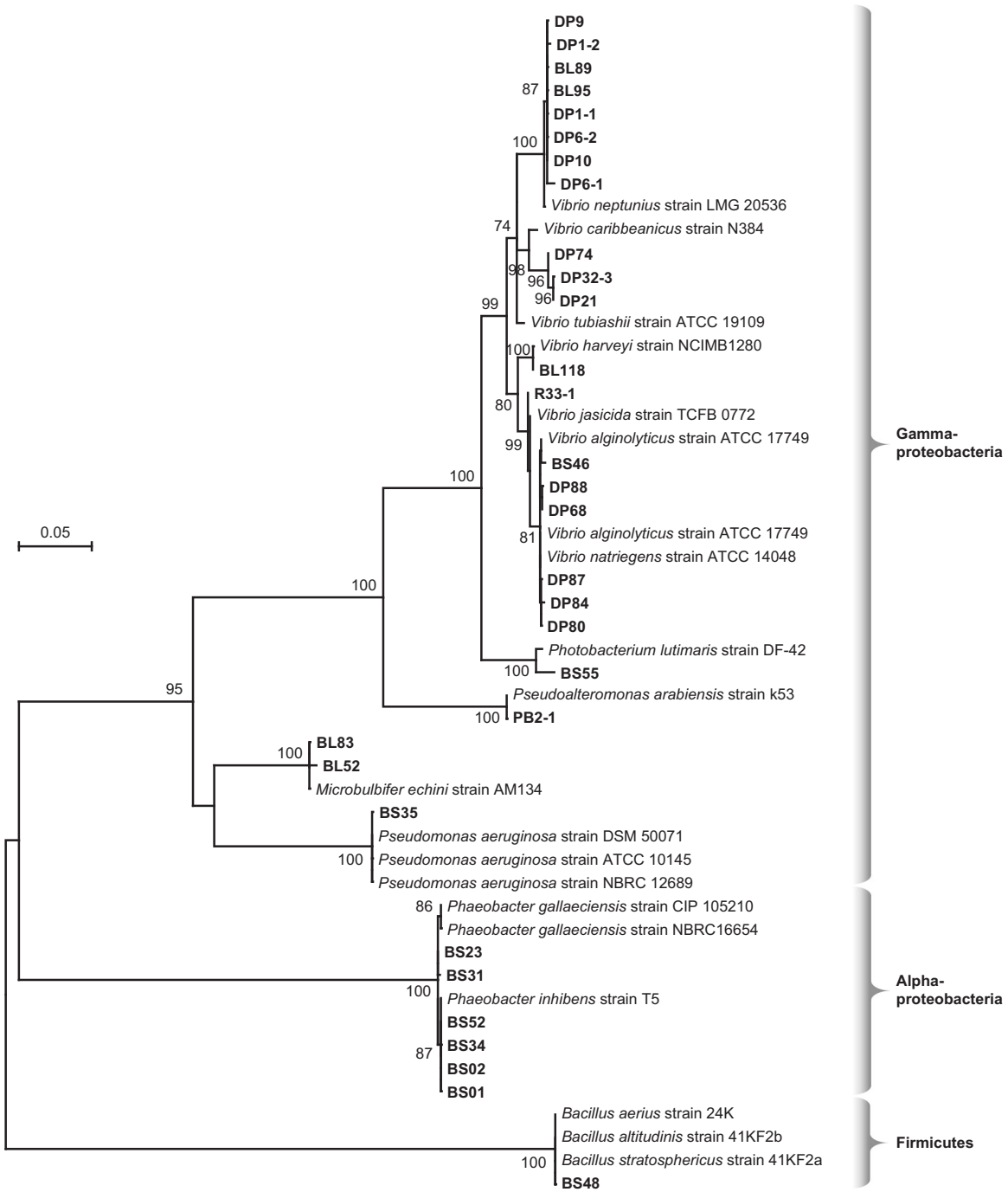

Fig. 2 Maximum likelihood tree constructed of the pathogen-antagonistic bacteria (bold) and the related sequences obtained from GenBank (July 2018). The tree is based on an alignment of near full-length 16S rRNA gene sequences (1119–1373 bp) and percentage bootstrap support (≥70% and based on 1000 replications) are given at the nodes. Scale bar shows the number of substitutions per site. The classes and phyla are shown on the right.

community differences, zOTUs were aggregated to different taxonomic levels (i.e. phylum to species). Bacteria associated with *D. pulchra* belonged to 18 classes, dominated by Gamma-proteobacteria (mean relative read abundance: 39.7%), Alpha-proteobacteria (37.1%) and Bacteroidia (22.4%). At the genus level, *Alteromonas* (13.1%), *Robiginitomaculum* (12.3%) and *Nonlabens* (11.8%) were the dominant taxa (Fig. S3). Further, the bacteria could be classified to 389 species, with only 19 species having a relative read abundance of ≥1% but accounting for >75% of the relative read abundance in the *D. pulchra* microbiome (Fig. S4). A significant effect of the treatment was detected only for the relative abundances of two species (i.e. with mean relative read abundance ≥1%), *Alteromonas alba* [58] (GTDB identifier *Alteromonas_A* sp002993365 and representing 56 zOTUs, Table S10; negative-binomial GLM, *Deviance* = 14.069, $p = 0.028$, Table S11) and *Cobetia marina* [59] (representing three zOTUs, Table S10; negative-binomial GLM, *Deviance* = 18.839, $p = 0.016$, Table S11) (Fig. 4). Further pairwise comparisons indicated that co-inoculation of BS52 + AD1, but not BS23 + AD1 significantly increased the relative abundance of the *A. alba* compared to the AD1-only treatment ($p_{adjusted} = 0.040$), with the relative abundance of this strain in the

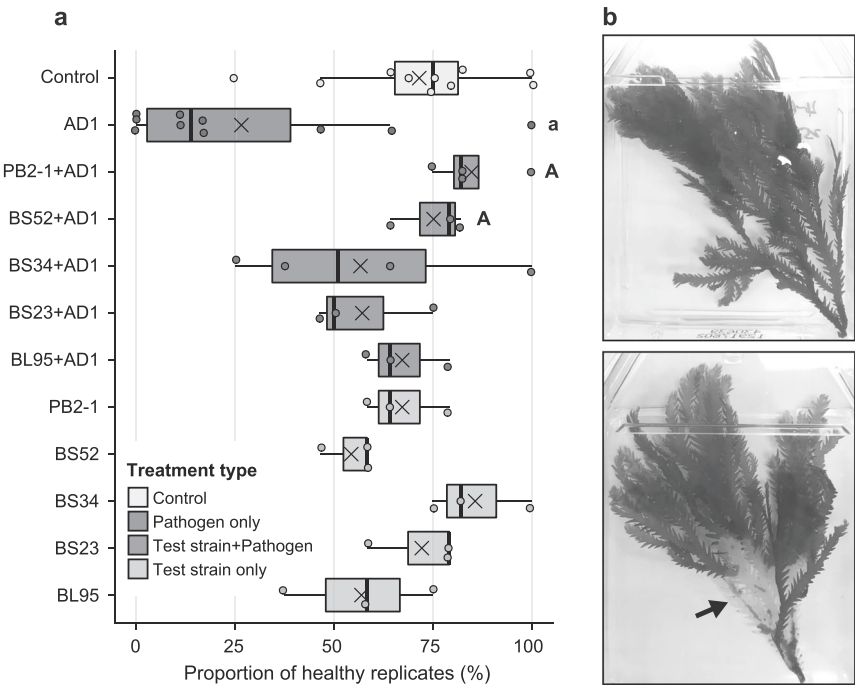

Fig. 3 Effect of bacterial treatments on *Delisea pulchra* health. a Proportion of healthy *D. pulchra* replicates in different treatments in independent replicate experiments. The x-axis shows the proportion of healthy replicates in each independent replicate experiment (each dot on graph represents one experiment, at least three are performed for each treatment, see Table S2 for detailed information). The cross represents the mean value calculated from replicate experiments. The y-axis shows the treatments: *D. pulchra* samples are treated by either the pathogen *Aquimarina* sp. AD1 (i.e. AD1, total number of replicates: $n = 58$), the candidate protective bacteria *Pseudoalteromonas* sp. PB2-1 (PB2-1, $n = 19$), *Phaeobacter* sp. BS52 (BS52, $n = 19$), *Phaeobacter* sp. BS34 (BS34, $n = 17$), *Phaeobacter* sp. BS23 (BS23, $n = 18$), *Vibrio* sp. BL95 (BL95, $n = 17$), the co-inoculation of one of the candidate protective bacteria and the pathogen, i.e. PB2-1 + AD1 ($n = 25$), BS52 + AD1 ($n = 20$), BS34 + AD1 ($n = 23$), BS23 + AD1 ($n = 17$), BL95 + AD ($n = 19$) or the Br-ASW as Control ($n = 58$). The statistical difference ($p < 0.05$) between single strain treatments (pathogen or test strain only) and the control is shown by a lowercase letter 'a', while differences between test strain +pathogen and the pathogen only treatments are denoted by an uppercase 'A'. **b** Representative images for the healthy (upper panel) and diseased (lower panel) *D. pulchra* in the infection assays. The disease was characterised by the localised loss of pigment around the mid-thallus (shown with the arrow).

BS52 + AD1 treatment being similar to that of the control (Table S11, Fig. 4). In addition, we found the AD1-alone treatment significantly increased the relative abundance of *C. marina* compared to controls ($p_{adjusted} = 0.046$). The co-inoculation of BS52 + AD1 had a lower relative abundance of this strain compared to AD1-only, but this trend was not significant ($p_{adjusted} = 0.145$, Table S11, Fig. 4).

DISCUSSION

Seaweed health, development and growth is influenced by the diverse bacterial communities they host [17, 60, 61]. However, despite bacterial symbionts being broadly recognised as important for host defense against pathogens [62], few studies have investigated the disease protective potential of seaweed-associated bacteria [13, 35]. Moreover, nothing is known about the underlying mechanisms or interactions that contribute to disease protection. To identify strains with potential disease-protective functions, we screened a collection of epiphytic bacteria for antagonistic activity towards known pathogens of *D. pulchra*. While antagonistic bacteria were identified from a range of taxa, bacteria affiliated with the genera *Phaeobacter*, *Vibrio* and *Pseudoalteromonas* showed the highest level of antibacterial activities and were sometimes even antagonistic against multiple pathogens from different taxa. This result is in line with an earlier study showing that seaweed-associated bacteria from these genera are potent antagonists for a range of gram-positive and/or gram-negative opportunistic pathogens of humans and animals [38]. Similarly, bacteria isolated from healthy coral mucus belonging to these taxa were reported to inhibit a broad range of other coral-associated bacteria including the pathogens *Vibrio coralliilyticus* and *Thallassomonas loyana* [63, 64], and a decline of antimicrobial *Vibrio* spp. and *Pseudoalteromonas* spp. within coral mucus during an ocean warming event coincided with increased coral bleaching [65]. Together these results further support the concept that the healthy microbiota of marine hosts possess pathogen suppressing strains that might mitigate disease in situ.

Inoculation with *Pseudoalteromonas* sp. PB2-1 and *Phaeobacter* sp. BS52 resulted in reduced levels of bleaching disease in *D. pulchra* upon pathogen challenge. There are several reports of the use of *Pseudoalteromonas* strains as potential probiotics in the aquaculture industry [66–68], including recent evidence suggesting *Pseudoalteromonas* species protect the red alga *Agarophyton vermiculophyllum* from tip bleaching [13]. *Phaeobacter* spp. are frequently isolated from marine aquaculture facilities, with studies demonstrating reduced pathogen loads and improved survival of pathogen challenged hosts, such as molluscs and fish larvae through the addition of selected *Phaeobacter* spp. to aquaculture tanks [69–74].

The ability to establish itself within a host microbiota is an important quality of a successful probiotic [75, 76]. Microbiome analysis of *D. pulchra* individuals after inoculation showed a significant increase in the relative abundances of *Phaeobacter* sp. BS52, demonstrating a successful bacterial delivery. It is noteworthy that sequences identical to *Phaeobacter* sp. BS52 were also detected (albeit in lower abundances) in uninoculated samples. This observation not only provides further support that *Phaeobacter* sp. BS52 is a naturally occurring member of *D. pulchra*'s microbiota, but also explains the successful establishment of this strain in the algal-

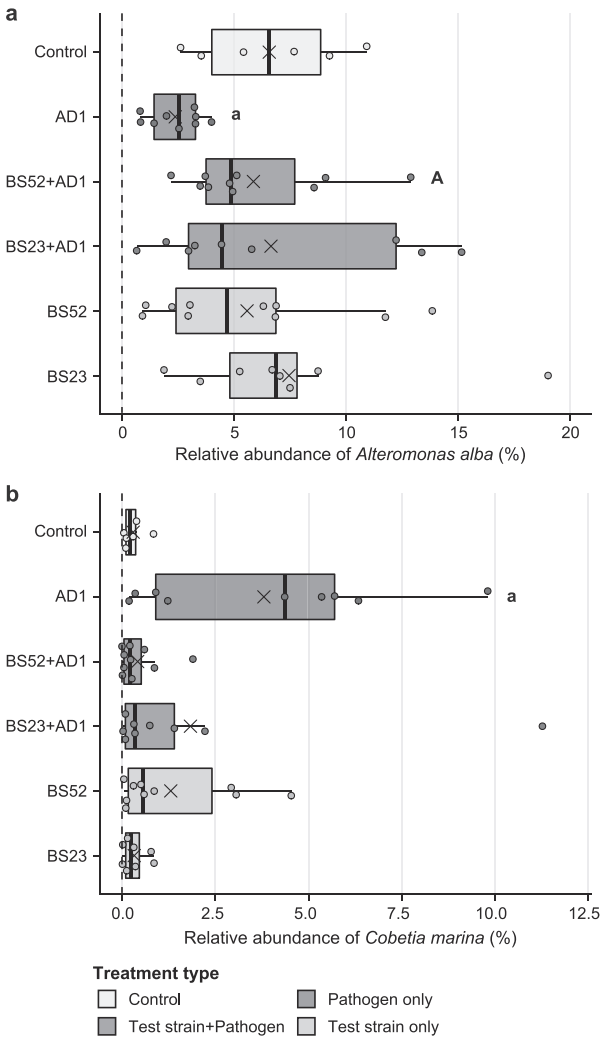

Fig. 4 Two bacterial species that had significantly different relative abundances in their bacterial communities (based on sequencing the 16S rRNA gene V3–4 regions) associated with *Delisea pulchra* of different treatments. The x-axes show the relative abundance of species: **a** *Alteromonas alba* (Family: *Alteromonadaceae*) and **b** *Cobetia marina* (Family: *Halomonadaceae*). The y-axes show the treatments: *D. pulchra* samples are treated with either AD1 ($n = 9$), BS52 + AD1 ($n = 10$), BS23 + AD1 ($n = 9$), BS52 ($n = 10$), BS23 ($n = 8$) or the Br-ASW as Control ($n = 6$) from three independent replicate experiments (see Table S6 for detailed replication information). The cross represents the mean value. A vertical reference line is drawn to indicate the zero relative abundance (i.e. absence). The statistical differences ($p < 0.05$) between single strain treatments (pathogen or test strain only) and the control are shown by a lowercase letter 'a', while difference between the test strain+pathogen and the pathogen only treatments is denoted by an uppercase 'A' (for details see Table S11).

associated microbial community. These results are consistent with observations based on other marine systems, e.g. in turbot larvae, microalgae and copepod nauplii [77], diatoms [78], and coccolithophores [79], where *Phaeobacter* spp. were strong natural niche competitors and could rapidly reach high relative abundances during artificial manipulation of the host microbiota.

However probiotic establishment is not always successful in marine systems. For example, despite being recognised as native to the host, increased relative abundances were not observed after the delivery of strains of *Pseudoalteromonas* sp., *Halomonas taeanensis* [34], *Acinetobacter* sp. [33], or *Halobacteriovorax* sp. [80] for corals. The fact that members of genus *Phaeobacter* have been repeatedly identified as potent antagonists and that they can be stably maintained within the host microbiota, suggests that they function as natural probiotics in marine systems. However, two strains phylogenetically closely related to *Phaeobacter* sp. BS52, namely *Phaeobacter* sp. BS23 and BS34, displayed no or only moderate protection (Fig. 3). Moreover, the *Phaeobacter gallaeciensis* LSS9 has been previously characterised as an opportunistic pathogen of *D. pulchra* [20]. These observations demonstrate that disease protective effects observed for the genus *Phaeobacter* very much depend on the species or even the specific strain. Differences in protective properties were also found in *Phaeobacter* strains when investigated for their potential as a probiotic for cod larvae. Specifically, D'Alvise et al. [81] challenged cod larvae with the pathogen *Vibrio anguillarum* and found that *Phaeobacter gallaeciensis* BS107 and *Phaeobacter* sp. M23-3.1 could reduce larval mortality, whereas a related strain *Phaeobacter* sp. 27-4 could not. Production of tropodithietic acid (TDA), which is known for its wide spectrum of inhibitory activity towards common marine pathogens [69, 73, 82], has previously been suggested as a contributing factor to strain difference in the protective traits of *Phaeobacter* species [83–86].

However, production of this antibiotic alone is unlikely to be the sole contributor to disease protection in *D. pulchra*, as both the protective (BS52) and the non-protective (BS23) *Phaeobacter* strains are capable of TDA synthesis (Supplementary information). Moreover, while *Phaeobacter* sp. BS52 directly inhibited the growth of *Aquimarina* sp. AD1 in an in vitro bioassay, we did not find evidence for a negative correlation in their relative abundances in vivo infection experiments, as would have been expected if direct antagonism occurred. One possible explanation for such observations could be that rather than destroying the pathogens, the protective strains might inhibit the activity or virulence of the pathogens—as has been suggested as one of the common modes of action for probiotics [87]. For example, Zhao *et al*. [88] reported that the oyster probiotics *Phaeobacter inhibens* S4Sm released secondary metabolites that hijack the ability of *Vibrio coralliilyticus* to communicate via quorum sensing, thereby downregulating transcription of genes involved in virulence.

A second possibility of disease protection is that the presence of *Phaeobacter* sp. BS52 influences other members of the macroalgal microbiota to the extent that it mitigates the dysbiotic effect of a pathogen. We found that the microbiota associated with *D. pulchra* individuals inoculated with *Phaeobacter* sp. BS52 and the pathogen *Aquimarina* sp. AD1 closely resembled those of the uninoculated samples and both were distinct from those exposed only to the pathogen. Specifically, co-inoculation with *Phaeobacter* sp. BS52 and the pathogen AD1 (BS52 + AD1) resulted in a significant increase in the relative abundances of multiple OTUs corresponding to *Alteromonas alba* [58] compared to the pathogen (AD1) only treatment, reaching levels similar to that of the uninoculated controls. Members of the genus *Alteromonas* have been reported as potential probiotics for several mariculture hosts and corals, as they have antibacterial and quorum quenching activities [89], including activity against pathogens like *Vibrio anguillarum*, *V. harveyi* and *Aeromonas hydrophilla* [90–92]. It is possible that *A. alba* plays a similar beneficial role for *D. pulchra*, and that the treatment with *Phaeobacter* sp. BS52 enhances its growth and thus indirectly reduces the pathogenic effects of *Aquimarina* sp. AD1. Conversely, compared to the pathogen-only treatment, a decrease in the relative abundance of three OTUs corresponding to *Cobetia marina* was observed when *Phaeobacter* sp. BS52 was inoculated in the presence of the pathogen (BS52 + AD1). Strains of *C. marina* have been reported to be associated with disease of the red alga *Porphyra* spp. [93]. *C. marina* strains are also dominant members of the microbial community associated

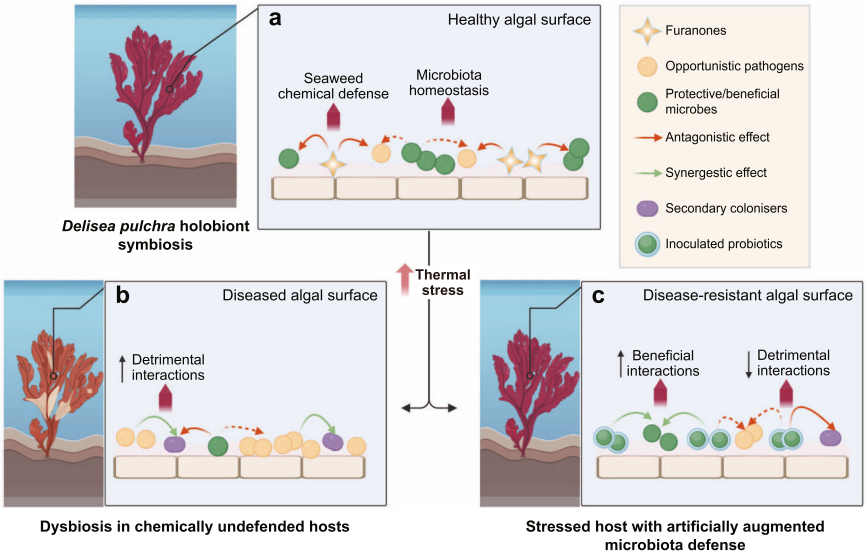

Fig. 5 A model for the bleaching disease pathogenesis of *Delisea pulchra* under a thermal stress and the concept of augmenting host disease resistance using probiotics. The solid lines represent experimentally evidenced interactions while dashed lines represent hypothesised processes. **a** Microbial interactions within a healthy *D. pulchra* holobiont are mitigated by the host chemical defense based on the release of furanones [98]. **b** Thermal stress can compromise host chemical defense and result in dysbiosis (increased detrimental interactions) in host microbiota, consequently inducing bleaching disease by opportunistic pathogens [12, 18–20, 22–24]. **c** We propose that probiotics can be used to promote beneficial and reduce detrimental interactions in host microbiota and increase disease defense in *D. pulchra* under suboptimal environmental conditions [Ref. [21] and current study].

with the degradation of the brown alga *Fucus evanescens* [94], and while they appear not to directly hydrolyse this alga's polysaccharides, they benefit from mono- and disaccharides released after the initial degradation [94, 95]. Given these previous observations, *C. marina* might have a role in accelerating disease progression by opportunistically scavenging on sugars or nutrients released from *D. pulchra* that has been impacted by the infection with *Aquimarina* sp. AD1, which is in line with the higher relative abundance of *C. marina* in the presence of AD1 compared to the control.

While it is possible that changes in the relative abundances of *A. alba* and *C. marina* are directly related to the action of the pathogen *Aquimarina* sp. AD1, our results show that the addition of *Phaeobacter* sp. BS52 can mitigate the microbiota shifts associated with AD1. Given previous work has suggested that disease of *D. pulchra* is partly a result of bacterial dysbiosis [9, 12], we propose that through maintaining *Alteromonas alba*, and through preventing the proliferation of the saprophyte *C. marina*, *Phaeobacter* sp. BS52 contributes (directly or indirectly) to the stability of the *D. pulchra* microbiota thus protecting it from the bleaching disease (Fig. 5). Such a role of probiotics on the homeostasis of host microbiota, through directly or indirectly reducing the negative effect of biotic/abiotic stress have also been reported in terrestrial plants [96] and humans [97] and the work here further expands these concepts to the marine environment.

DATA AVAILABILITY

The sequence data has been submitted to the BioProject database under accession numbers PRJNA630012. Electronic supplementary information is available through: https://doi.org/10.6084/m9.figshare.14815191.

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AUTHOR CONTRIBUTIONS

JL, TT, and SE designed the study. JL and ZD performed the experiments. MEM, JL, and EMM performed the data analyses. JL and SE wrote the first draft of the manuscript. All authors contributed to the writing of the final version of the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

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