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Research Paper

Validating the use of ROS-scavenging bacteria as probiotics to increase coral resilience to thermal stress*

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Abstract Thermal stress causes the overproduction and toxic accumulation of reactive oxygen species (ROS), which seems to be correlated with coral bleaching and, ultimately, death. The reduction of ROS concentration within the coral holobiont could minimize the effects of thermal stress and support efforts to reduce coral decline globally. In the current study, we explored the physiological responses of *Pocillopora damicornis* to ROS-scavenging bacteria inoculation as well as the microbiome restructuring that correlates with *P. damicornis*'s resilience to thermal stress after probiotic inoculation. Inoculation of corals with ROS-scavenging bacteria enhanced coral health and reduced ROS concentration. Furthermore, the enhanced coral thermal resistance promoted by ROS-scavenging bacteria was also correlated with an overall coral microbiome restructuring. In addition, the complex network relationships between bacteria and Symbiodiniaceae in corals after ROS-scavenging bacteria inoculation contributed to corals' resilience to high temperatures. Besides, coral heat tolerance bacterial biomarkers, such as Myxococcota, were enriched in corals with added ROS-scavenging bacteria. Collectively, our findings validate the selected ROS-scavenging bacteria as coral probiotics that could help corals resist thermal stress on a short timescale. Additionally, our data contribute to our understanding of the potential interactions between different members of the coral holobiont and the use of probiotics as tools to aid coral restoration efforts.

Keyword: coral; beneficial microorganisms for corals (BMCs); Symbiodiniaceae; thermal stress; coral bleaching

1 INTRODUCTION

Coral reefs are one of the most important ecosystems on earth and are generally described as tropical rainforests in the ocean because of their high primary productivity and biodiversity (Silveira et al., 2017). However, global warming and local anthropogenic pressures on coral reefs are exposing corals to severe stress, leading to mass bleaching, disease, and mortality (Eddy et al., 2021). There has been a growing consensus that coral bleaching

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caused by rising ocean surface temperatures is one of the predominant reasons for the massive decline of coral reefs around the world in the last 30 years (Hughes et al., 2017; Oliver et al., 2018; Knowlton et al., 2021). Consequently, increasing attention has focused on the protection and restoration of coral reefs to mitigate this loss. Furthermore, assisted evolution (van Oppen et al., 2015), bioengineering tools (Curran and Barnard, 2021), microbiome transplantation (Doering et al., 2021), coral probiotics (Peixoto et al., 2017; Rosado et al., 2019; Santoro et al., 2021; Zhang et al., 2021b), and restoration methods (Shaver et al., 2022) have been developed to prevent coral bleaching and mortality and to maintain the persistence of coral reefs.

Coral harbors diverse and dynamic microbial communities, including bacteria, fungi, viruses, Symbiodiniaceae, and archaea, which together are termed as the coral microbiome (Blackall et al., 2015). Changes in coral microbiome composition were observed in stressed, diseased, and bleached corals (Zaneveld et al., 2016; Patel et al., 2021). Photosynthates derived from the endosymbiont Symbiodiniaceae are the main source of energy for the coral host (Falkowski et al., 1984). Efficient recycling of nutrients between coral and Symbiodiniaceae is the key to the symbiosis of coral-algal symbionts (Muscatine and Porter, 1977). Therefore, coral health and their ability to resistant environmental disturbance rely on the nutrient input provided by algal symbionts (Lesser, 2013). Heat stress destabilizes the symbiotic nutrient cycling within the coral holobiont, which may trigger the breakdown of the coral-algal symbiosis (Rädecker et al., 2021). Moreover, thermal stress can lead to photoinhibition of photosynthesis in the Symbiodiniaceae (Iglesias-Prieto et al., 1992). Photoinhibition occurs when photosynthetic electron transport is reduced, excitation energy is absorbed rapidly, and reactive oxygen species (ROS) are produced (Lesser, 2006). ROS are composed of superoxide anion, hydrogen peroxide, hydroxyl radical, and singlet oxygen (Borisov et al., 2021). Although ROS can be converted back to oxygen and water by ROS handling enzymes such as catalase and ascorbate peroxidase (Asada and Takahashi, 1987; Halliwell and Gutteridge, 2015). More ROS are formed under heat stress than can be quenched by available enzymatic and nonenzymatic antioxidants (Lesser, 2006). The overproduction and toxic accumulation of ROS may permanently damage the host and symbiont organelles (Weis, 2008; Roberty et al., 2015; Suggett and Smith, 2020; Al-Hammady et al., 2022). Breakdown of the symbiosis of coral hosts and Symbiodiniaceae results in coral hosts exhibiting a series of cellular responses, including apoptosis, exocytosis, and autophagy, through which the Symbiodiniaceae can be eliminated or expelled, culminating in coral bleaching (Weis, 2008; van de Water et al., 2018; Li et al., 2021). Therefore, tools to mitigate ROS within the coral holobiont may minimize the physiological damage caused by thermal stress (Peixoto, 2017; Dungan et al., 2021). Furthermore, coral bleaching has been shown to be closely related to the dysbiosis of the coral microbial community (Sweet et al., 2017; West et al., 2019). Thus, the selection and retention of probiotic bacteria can delay or mitigate dysbiotic processes, increasing resilience (Peixoto and Voolstra, 2023).

Recently, the term “Beneficial Microorganisms for Corals” (BMC) has been proposed to define coral probiotics that promote coral health and development, and microbiome engineering through the addition of BMCs is suggested as a strategy to enhance the resilience of corals to disturbances (Peixoto et al., 2017, 2021). Microbes capable of scavenging ROS were observed to potentially relieve coral thermal stress (Tandon et al., 2022). Thus, ROS-scavenging bacteria may be beneficial for corals, as they may increase corals’ resilience to heat stress (Motone et al., 2020). In the current study, the potential beneficial effects and mechanisms of the addition of ROS-scavenging bacteria on the resilience of coral against heat stress were explored. Such studies on beneficial ROS-scavenging bacteria in corals may facilitate the development and application of microbiome engineering in coral reef restoration.

2 MATERIAL AND METHOD

2.1 Bacteria selection and identification, catalase assay, and whole genome sequence analysis

The bacterial strain FRS1 was previously isolated from *Acoropora digitifera* coral. Briefly, 0.5 g of coral sample was homogenated and resuspended in 50 mL of sterile filtered seawater and then shaken for 48 h. After enrichment culture, 10^{-5} dilution of the medium was inoculated into petri dishes containing 1% NaCl Luria-Bertani (LB) medium (5 g of tryptone, 2.5 g of yeast extract, 5 g of NaCl, and 9 g of agar in 500-mL seawater). All plates were cultured at 28 °C for 48 h, and single colonies with uniform morphology were selected. 16S rRNA gene sequence of the strain was amplified with primer

pairs 27F and 1492R (Galkiewic and Kellogg, 2008), and the PCR products were used for sequencing. The obtained sequence was analyzed through BLAST programs (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Then, strain FRS1 was frozen at -80 °C with 25% glycerin. Strain FRS1 was verified for ROS-scavenging potential based on a qualitative assay. Briefly, 30-mL liquid culture of strain FRS1 was mixed with 30 μL of 3% (v/v) hydrogen peroxide (Santoro et al., 2021) and the mixture was observed for the presence of bubbles; the appearance of bubbles indicated that the organism was catalase-positive.

In addition, whole genome sequencing of strain FRS1 was conducted for molecular analysis. Total bacterial DNA was extracted from a single colony of strain FRS1 using a Wizard Genomic DNA Kit (Promega, Whitehead Scientific, CT, South Africa). High-throughput sequencing was performed on Illumina platforms or the PacBio RSII platform at Novogene (Tianjin, China). The resulting FASTQ reads were quality filtered with Trimmomatic (v0.36) (Bolger et al., 2014), and bases were removed at the tailing end if the PHRED score <30. The filtered subreads were assembled by Canu V1.5 software (Koren et al., 2017), and then Circlator v1.5.5 (Hunt et al., 2015) was used to circulize the assembled genome. The GenBlastA v1.0.4 (She et al., 2009) program was used to scan the whole genome after masking predicted functional genes. Functional annotation of coding genes was performed with Prodigal v2.6.3 (Hyatt et al., 2010). Predicted gene sequences were blasted against Nr, Swiss-Prot, TrEMBL, KEGG, and eggnog databases by Blast v2.2.29, followed by Blast2go (Conesa et al., 2005) for Gene Ontology (GO) annotation. GenomeView (Abeel et al., 2012) was used to screen hydrogen peroxide metabolism and superoxide radical removal genes in strain FRS1.

2.2 Coral collection and experimental design, and the addition of ROS-scavenging bacteria

Coral sampling was permitted by the Department of Agriculture and Rural Areas of Hainan Province. Ten colonies of *P. damicornis* were collected from the Luhuitou fringing reef in Sanya, China (18°12′N, 109°28′E), on November 7, 2020. Corals were collected from a depth of 1.5–2 m. The seawater temperature was 27–28 °C, and the salinity was approximately 34. The coral colonies were divided into 48 fragments (approximate diameter 3–5 cm) and fastened to a round ceramic base. The fragments were acclimated for 7 d in the breeding shed and then moved indoors for the cultivation experiment (Supplementary Fig.S1). Before inoculation, strain FRS1 was recovered and fermented in 250-mL LB medium at 28 °C for 48 h. Bacterial cell counts were estimated using a hemocytometer under a light microscope. When the strain reached 1×10^7 cells/mL, the culture was centrifuged at 5 000×*g* for 10 min. The cell pellet was washed three times with 0.22-μm filtered seawater (FSW) and resuspended in 25-mL FSW to 1×10^8 cells/mL.

The mesocosm experiment was conducted at two temperature regimes (28 °C and 32 °C) and three treatment regimes (Supplementary Fig.S1): blank control samples (NT) without inoculation FRS1 (28 °C), heat stress control samples (HT) without inoculation FRS1, and heat stress with strain FRS1 inoculation samples (BT). Each group comprised six tanks. Therefore, there were 18 tanks in total for the three groups. A temperature controller was installed in each tank, and the initial temperature of all tanks was 28 °C; the temperature of the BT and HT tanks (12 tanks in total) slowly rose to 32 °C within 1 week (about 1 °C per day) and was subsequently maintained at 32 °C for 14 d. There was 0.5-μm FSW circulating in the tanks, and the flow rate was 15 L/h. The light intensity above the plastic tanks was 200 μmol photons/(m^2·s) provided by an aquarium light in a 12-h:12-h light/dark cycle. The 48 coral fragments were randomly distributed among three groups: NT (*n*=18), HT (*n*=18), and BT (*n*=12). Some fragments were used as test samples before the addition of bacteria, hence the large number of fragments in the NT and HT groups. Strain FRS1 was added to six tanks after the temperature of the tanks reached 32 °C on day 7; the concentration of bacteria in the tanks was 1×10^5 cells/mL. After addition of bacteria, the water in the tanks was kept static for 24 h. The whole experiment process included three samplings: before bacteria addition, 1 week after bacteria addition, and 2 weeks after bacteria addition, respectively. Since the BT tank is the same as the HT tank before adding bacteria, the BT tank is not sampled before adding bacteria. For sampling, one coral fragment was randomly taken from each of the six tanks, a total of six replicates per group for each sampling. Coral fragments were removed from the tanks with sterile forceps before being ground in a sterilized pre-cooled mortar. Each replicate was then equally dispensed into five cryogenic vials and immediately snap frozen and stored in liquid nitrogen until analysis. In three groups, 16S rRNA and ITS genes metabarcoding were used to investigate microbiome

changes associated with corals (Days 7, 14, and 21). At each time point, the potential beneficial effects of the strain FRS1 were assessed through the evaluation of coral physiology, including photosynthetic efficiency of Symbiodiniaceae (F_v/F_m), protein content, Symbiodiniaceae density, and chlorophyll content.

2.3 Assessment of the physiological parameters of coral

Six coral replicates per treatment were photographed, and the color of the coral tissues was assessed through the Coral Color Reference Card developed for standardizing changes in coral color at each time point (Siebeck et al., 2006). Before each sampling time point, the photosystem II photochemical efficiency of Symbiodiniaceae was assessed using pulse-amplitude-modulated fluorometry as a proxy for coral holobiont health. Photosystem II activities of corals were determined by the maximum quantum yield (F_v/F_m) of photochemistry. The minimum (F_0) and maximum (F_m) fluorescence levels were measured to calculate the fluorescence variables (F_v) using a diving-PAM fluorometer fitted with a red-emitting diode (Walz, Germany). Four coral nubbins were randomly selected from each group for determination of photosynthetic activity. The diving-PAM settings were as follows: saturating pulse intensity (SI)=8, measuring light intensity (MI)=7, saturating pulse width (SW)=0.8, gain (G)=7, and damping (D)=3.

Triplicate coral samples per treatment were collected to determine protein content, Symbiodiniaceae density and chlorophyll-*a* (chl-*a*) content. The protein content of coral samples was measured using a Detergent Compatible Bradford Protein Assay Kit (Beyotime, China), with three replicates per group determined at each time point. Symbiodiniaceae cell density was measured according to Rossi et al. (2020). Briefly, approximately 0.5-g coral sample was transferred into a 10-mL centrifuge tube, then 5-mL FSW was added and mixed well. The homogenates were subsequently filtered with 20-μm nylon filters and stood for 30 s, and then the resulting supernatant was transferred into a new tube and centrifuged at 4 °C, 3 000×*g* for 2 min. The supernatant was removed, and the precipitant was resuspended in 500-μL FSW. Ten microliters of the resulting tissue homogenate were applied to a hemocytometer and counted six times. To determine chlorophyll *a*, samples were treated as for Symbiodiniaceae density and then extracted with 100% acetone at 4 °C for 24 h. The absorbance of the extracts was measured at wavelengths of 630, 647, 663, and 750 nm, and chlorophyll-*a* content was calculated from the corresponding equation (Jeffrey and Humphrey, 1975). About 1 g of each coral sample was dried for 48 h in a drying oven at 60 °C to determine dry weight. Then, dried samples were transferred into a pre-weighed crucible and combusted at 450 °C in a muffle furnace for 4 h. Combusted samples were cooled to room temperature and weighted (ash weight). Subsequently, ash-free dry weight was calculated by subtracting ash weight from dry weight. All the above parameters were normalized to ash-free weight (AFDW).

Three coral replicates per treatment were used to measure ROS content with a CM-H_2DCFDA fluorescent probe at each sampling time point. Reconstituted CM-H_2DCFDA was made from DMSO treated samples from the same treatment. To test the probe's efficiency, hydrogen peroxide was added to the coral sample as a positive control. Coral fragments were transferred to 50-mL centrifuge tubes, then 5-mL FSW was added, and the bottom of the tube was tapped to remove the tissues. Exfoliated coral tissues were transferred into 10-mL centrifuge tubes and centrifuged at 25 °C, 3 000×*g* for 1 min. The resulting supernatant was discarded, and the precipitate was washed three times with FSW to remove non-symbionts. The precipitate obtained after washing was resuspended in 2-mL FSW and reacted with 5-μL CM-H_2DCFDA in the dark for 30 min. The reactant was then washed twice with FSW and resuspended in 1-mL FSW for confocal microscopy (Olympus FV3000, Japan) detection. Red dots indicate Symbiodiniaceae without ROS, and green dots indicate Symbiodiniaceae invaded by ROS. The excitation and emission wavelengths for confocal microscopy were 488 nm and 525 nm, respectively. A photograph of ROS was processed with Fiji software (Schindelin et al., 2012), and the number of green dots was counted for statistical analysis.

2.4 DNA extraction, polymerase chain reaction (PCR), and sequencing

Microbial community genomic DNA was extracted from six coral samples using the E.Z.N.A.® soil DNA kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. All DNA samples were quality checked and the concentration was quantified by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Negative controls (no sample template) were also extracted and amplified under the same conditions. Bacterial 16S rRNA

gene fragments (V3–V4) were amplified from the extracted DNA using primers 338F (5′-ACTCCTAC GGGAGGCAGCAG-3′) and 806R (5′-GGACTACH VGGGTWTCTAAT-3′), while the ITS2 region of the ITS gene was amplified with the primer pairs ITS2F (5′-GAATTGCAGAACTCCGTG-3′) and ITS2R (5′-GGGATCCATATGCTTAAGTTCAGCGGGT-3′). The PCRs were performed with 4 μL 5× *TransStart* FastPfu buffer, 2 μL of 2.5-mmol/L deoxynucleoside triphosphates (dNTPs), 0.8-μL each primer (5 μmol/L), 0.4-μL *TransStart* FastPfu DNA Polymerase, 10 ng of DNA, and dH_2O to a volume of 20 μL. Negative controls used the same amount of dH_2O instead of DNA. The PCR conditions comprised 27 cycles of 30 s at 95 °C, 30 s at 55 °C, and 45 s at 72 °C. Agarose gel electrophoresis was performed to verify the size of the amplicons. Amplicons were then subjected to paired-end sequencing on an Illumina MiSeq sequencing platform using PE300 chemical at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

After demultiplexing, the resulting amplicon sequences were merged with FLASH (v1.2.11) and quality filtered with Fastp (0.19.6) (Magoč and Salzberg, 2011; Chen et al., 2018). High-quality sequences were then denoized using the DADA2 (Callahan et al., 2016) plugin in the QIIME 2 (version 2020.2) pipeline with recommended parameters, which obtains single nucleotide resolution based on error profiles within samples. DADA2 denoized sequences are usually termed amplicon sequence variants (ASVs). To minimize the effects of sequencing depth on alpha- and beta-diversity measures, the number of sequences from each sample was rarefied to 4 000, which still yielded an average Good's coverage of 97.90%. Bacterial sequences were classified using the SILVA database (v. 138), and Symbiodiniaceae sequences were assigned using the SymTyper pipeline (Edmunds et al., 2014), respectively. In total, 2 056 187 bacterial and 2 547 386 Symbiodiniaceae high-quality reads from 48 samples were retrieved and sorted into 14 367 and 102 ASVs, respectively. Both bacterial and Symbiodiniaceae alpha- and beta-diversity were calculated in QIIME 2. To confirm their presence within the coral microbiome, we sequenced the amplicons of our inoculant bacteria FRS1.

2.5 Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate the difference in the proportion of ROS among the three groups. Nonparametric statistical tests (Kruskal-Wallis test or Wilcoxon test) were performed to evaluate the difference in alpha diversity among the three groups and the difference in taxonomy between two groups. ANOSIM, Adonis, and MRPP analyses were performed to evaluate the difference in beta diversity between two groups. To assess the relative contribution of stochastic and deterministic processes to the coral-associated microbiome assembly, the beta Nearest Taxon Index (βNTI) was calculated through null model analysis (999 randomizations) (Stegen et al., 2013), defining $|\beta NTI| \geq 2$ as dominant deterministic processes and $|\beta NTI| \leq 2$ as dominant stochastic processes. In addition, stochastic and deterministic processes were divided into five ecological processes based on the values of both βNTI and Bray-Curtis-based Raup-Crick Index (RC_{Bray}), including homogeneous selection ($\beta NTI > +2$), dispersal limitation ($|\beta NTI| < 2$ and $RC_{Bray} > 0.95$), homogenizing dispersal ($|\beta NTI| < 2$ and $RC_{Bray} < -0.95$), undominated ($|\beta NTI| < 2$ and $|RC_{Bray}| < 0.95$), and heterogeneous selection ($\beta NTI < -2$) (Zhou et al., 2014; Jiao et al., 2020).

Microbial network analysis at the bacterial phylum level was performed using the R package "vegan" based on Spearman correlation scores (Spearman's $R > 0.6$ or $R < -0.6$; $P < 0.05$), and the same methodology was employed for the analysis of the co-occurrence network of bacterial and Symbiodiniaceae taxa. Both bacterial and Symbiodiniaceae ASVs present in all samples were retained for the network analysis, and the networks were visualized in Gephi (Dalcin and Wyse Jackson, 2018). Linear discriminant analysis (LDA) effect size (LEfSe) was applied (Kruskal P-value<0.05, logarithmic LDA score >2) to identify biomarker ASVs for specific groups. All statistical analyses were performed in R 4.1.1 (http://www.r-project.org).

3 RESULT

3.1 The ROS-scavenging potential of strain FRS1

The bacterial strain FRS1 was previously isolated from *Acoropora digitifera* coral, and it exhibited 98.58% similarity to a strain taxonomically classified as *Microbacterium zeae* (Supplementary Fig.S2). Using the catalase activity assay, the bacterial strain FRS1 showed potential for scavenging ROS. The draft genome sequence of strain FRS1 was assembled and analyzed, and genes involved in hydrogen peroxide scavenges (*katG*) and superoxide radical removal (*trxB*) were found in the genome of strain FRS1 (Supplementary Table S1). A lag phase-grown strain FRS1 was collected and resuspended in sterile sea water at 1×10^8 cells/mL.

3.2 Physiological parameter of coral

According to the coral color reference card, the coral color of all groups started at D6. The corals maintained at 28 °C displayed no visible color shift during the whole experiment (Supplementary Fig. S3). However, corals maintained at 32 °C showed varying degrees of bleaching. On Day 14, the color of corals in the HT group (32 °C, no added bacteria) decreased to D4, and on Day 21, the coral color declined to D1. The coral color in the BT group (32 °C, with added ROS-scavenging bacterial strain FRS1) was D5 on Day 14 and D4 on Day 21. The photosynthetic efficiency (F_v/F_m) of corals at 28 °C was significantly higher throughout the experiment compared with those at 32 °C (P<0.05, nonparametric Kruskal-Wallis test, Fig.1a). The photosynthetic efficiency of the HT (0.384±0.109) and BT (0.541±0.017) groups decreased by 41.91% and 18.15%, respectively, compared with the NT (0.661±0.002) group (28 °C, no addition of bacteria) at the end of the experiment. Furthermore, the addition of ROS-scavenging bacteria (BT group) markedly increased the photosynthetic efficiency in comparison with the HT group on Day 14 and Day 21 (P<0.05, nonparametric Kruskal-Wallis test). The protein contents of the BT and NT groups were also significantly higher than that of the HT group on Day 21 (P<0.05, nonparametric Kruskal-Wallis test, Fig.1b). During the experiment, the protein content in the HT and BT groups decreased by 57.01% and 2.95%,

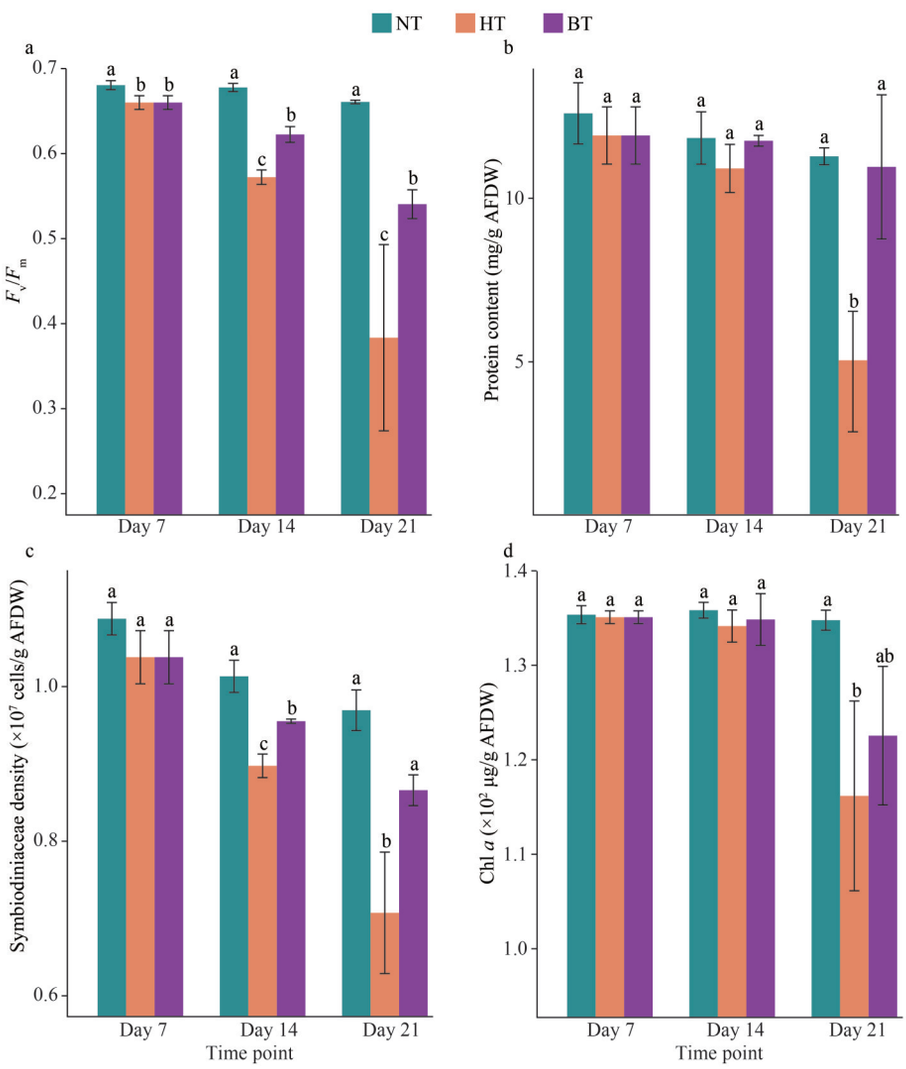

Fig.1 Physiological parameters of *Pocillopora damicornis* throughout the experiment

a. photosynthetic efficiency; b. protein content; c. Symbiodiniaceae density; d. chlorophyll-*a* content. Error bars represent means±SE (n=3). NT indicated corals maintained at 28 °C, and HT indicated corals maintained at 32 °C without bacterial inoculation, and BT indicated corals maintained at 32 °C with bacterial inoculation. Different letters above the bars indicate a significant difference determined by nonparametric Kruskal-Wallis test.

respectively, compared with that of the NT group.

There were no significant differences between corals maintained at 28 °C and 32 °C on Day 7 in Symbiodiniaceae density or chlorophyll-*a* content (P>0.05, nonparametric Kruskal-Wallis test, Fig.1c & d). However, on Day 14, the Symbiodiniaceae density in the NT and BT groups was significantly higher than that in the HT group (P<0.05, nonparametric Kruskal-Wallis test), but the chlorophyll-*a* contents in all three groups were similar. On Day 21, the Symbiodiniaceae density was significantly different between the HT and BT groups (P<0.05, nonparametric Kruskal-Wallis test).

3.3 ROS production within Symbiodiniaceae

The proportion of Symbiodiniaceae enriched with ROS in coral holobionts of the NT group remained constant throughout the experiment (Fig.2), and no significant difference was observed between the three groups on Day 7 (Fig.2). On Day 14 and Day 21, the proportion of Symbiodiniaceae enriched with ROS were significantly higher in the HT group than in the NT and BT groups. In addition, after thermal stress, the proportion of Symbiodiniaceae enriched with ROS in corals of HT group was much higher than that in the BT group. It appears that ROS-scavenging bacteria contribute to ROS removal in coral holobionts.

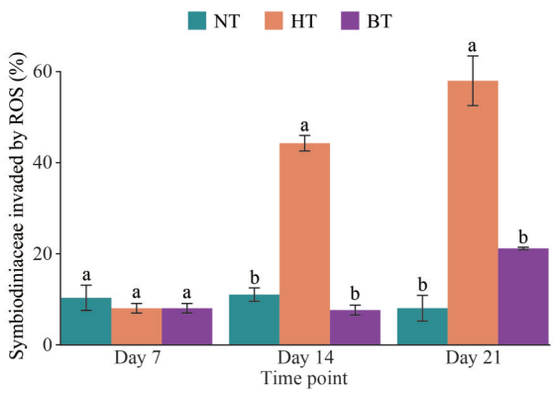

Fig.2 The proportion of Symbiodiniaceae enriched with ROS among three groups at each time point

Different letters indicate a significant difference measured by one-way analysis of variance (ANOVA).

3.4 Diversity and community assembly of bacteria and Symbiodiniaceae

Shannon indexes of bacterial and Symbiodiniaceae communities in the NT and HT groups remained constant over 3 weeks of the experiment (Fig.3; Supplemental Table S2). However, for the BT group, the Shannon index of the bacterial community

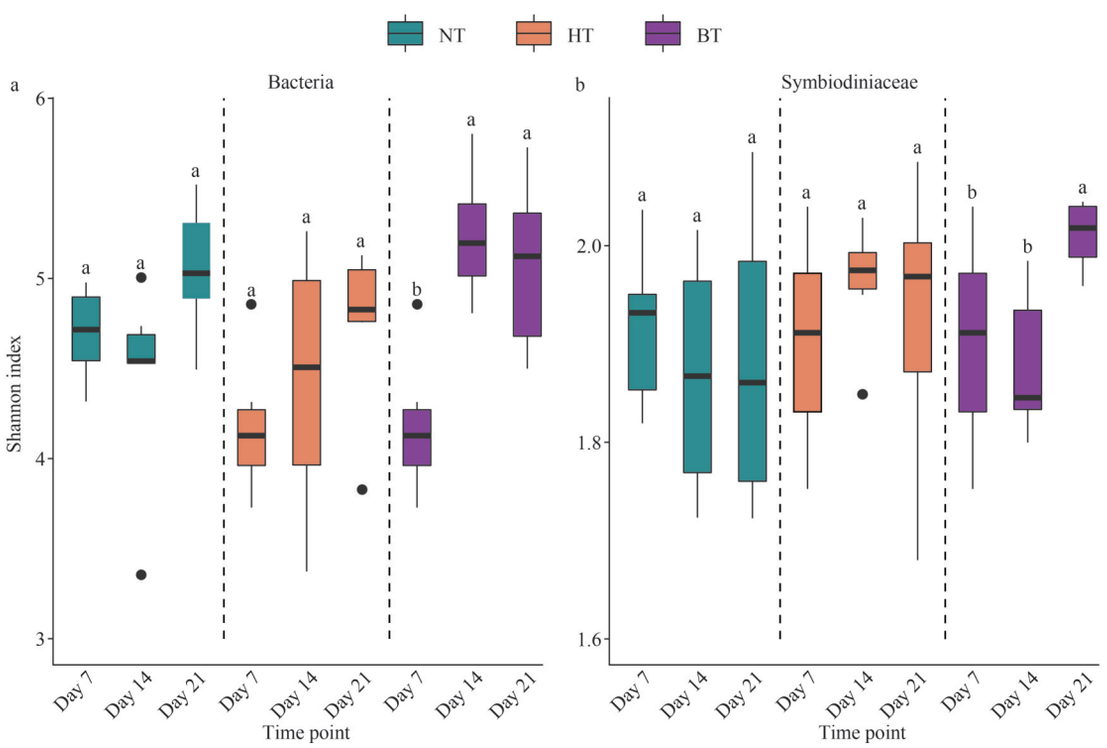

Fig.3 Alpha diversity of bacterial (a) and Symbiodiniaceae (b) communities associated with corals from each group (NT, HT, and BT) across 3 weeks

Different letters above the boxes indicate a significant difference determined by nonparametric Kruskal-Wallis test.

increased significantly on Day 14 and Day 21 compared with that on Day 7. However, there was no significant difference between Day 14 and Day 21. The Shannon index of the Symbiodiniaceae community in the BT group was not significantly different between Day 7 and Day 14 but increased significantly on Day 21. Moreover, other measures of bacterial alpha diversity, including observed amplicon sequence variants (ASVs), Chao, and Simpson indexes in the BT group showed a similar pattern to the Shannon index. However, for the Symbiodiniaceae community in the BT group, all the indexes but the Simpson index, were opposite to the pattern of the Shannon index.

The results of three nonparametric tests (analysis of similarity (ANOSIM), permutational multivariate analysis of variance (Adonis), and multiple-response permutation procedure (MRPP)) indicated that the bacterial and Symbiodiniaceae community dissimilarity was inconsistent at different stages. For the Symbiodiniaceae community, there was no significant dissimilarity among the three groups during the whole experiment (Table 1). However, the bacterial community presented a different profile. Although the bacterial communities of corals in the NT and HT groups were similar on Day 7 and Day 14, there was a significant difference between them on Day 21 (Adonis, P=0.018). In addition, there was a significant difference in the bacterial community between the HT and BT groups on Day 14, but this difference disappeared by Day 21. Principal co-ordinates analysis results were congruent with those of the ANOSIM, Adonis, and MRPP dissimilarity tests (Supplementary Fig.S4). Regression analysis based on beta-diversity partitioning showed that the changes in bacterial communities among all groups were predominantly driven by species turnover (Supplementary Fig.S5). For the dissimilarity of Symbiodiniaceae communities, the NT group was predominantly explained by species turnover throughout the experiment. Changes in the HT and BT groups were initially driven by species turnover but switched to species nestedness in the later stages of the experiment.

Null model analysis revealed that all coral samples exhibited stochastic bacterial community assembly (Fig.4a & b). On Day 14, the bacterial community of the HT group was driven by homogenizing dispersal and undominated. In contrast, the BT group was driven by homogenizing dispersal, dispersal limitation, and undominated. On Day 21, bacterial community assembly in all corals was driven by dispersal limitation, homogenizing dispersal, and undominated. Assembling Symbiodiniaceae communities in both NT and HT groups depended solely on stochastic processes, while in BT both stochastic and deterministic processes were involved (Fig.4).

A phylum-level bacterial taxonomic classification showed that Proteobacteria, Acidobacteriota, and Patescibacteria had consistently increased abundances (Fig.5a). On Day 14, the HT group had significantly lower abundances of Chloroflexi and Myxococcota than did the BT group (Wilcoxon test, P<0.05). On Day 21, Cyanobacteria and Fusobacteriota abundances in the NT and BT groups were significantly lower than those in the HT group on Day 21 (Wilcoxon test, P<0.05). However, Actinobacteriota abundances in the NT group and Proteobacteria abundances in the BT group were considerably higher than those in the HT group. *Rhodococcus* dominated the bacterial community at the genus level (Fig.5b). In all three

Table 1 Three different permutation tests (ANOSIM, Adonis, and MRPP) were performed based on Bray-Curtis distance

	Day	Group	ANOSIM		Adonis		MRPP	
			r	P	F	P	δ	P
Bacteria	7	NT vs. HT	0.117	0.135	0.136	0.110	0.024	0.131
	14	NT vs. HT	0.113	0.097	0.106	0.149	0.009	0.193
		BT vs. HT	0.535	**0.012**	0.249	**0.008**	0.096	**0.014**
	21	NT vs. HT	0.272	**0.015**	0.167	**0.018**	0.046	**0.020**
		BT vs. HT	0.135	0.087	0.136	0.087	0.035	0.060
Symbiodiniaceae	7	NT vs. HT	-0.119	0.936	0.023	0.836	-0.064	0.995
	14	NT vs. HT	0.034	0.292	0.081	0.285	-0.009	0.316
		BT vs. HT	0.220	0.083	0.215	0.078	0.083	0.105
	21	NT vs. HT	0.070	0.202	0.153	0.192	0.048	0.161
		BT vs. HT	0.027	0.291	0.107	0.332	0.013	0.273

Bold values indicate test results with P<0.05.

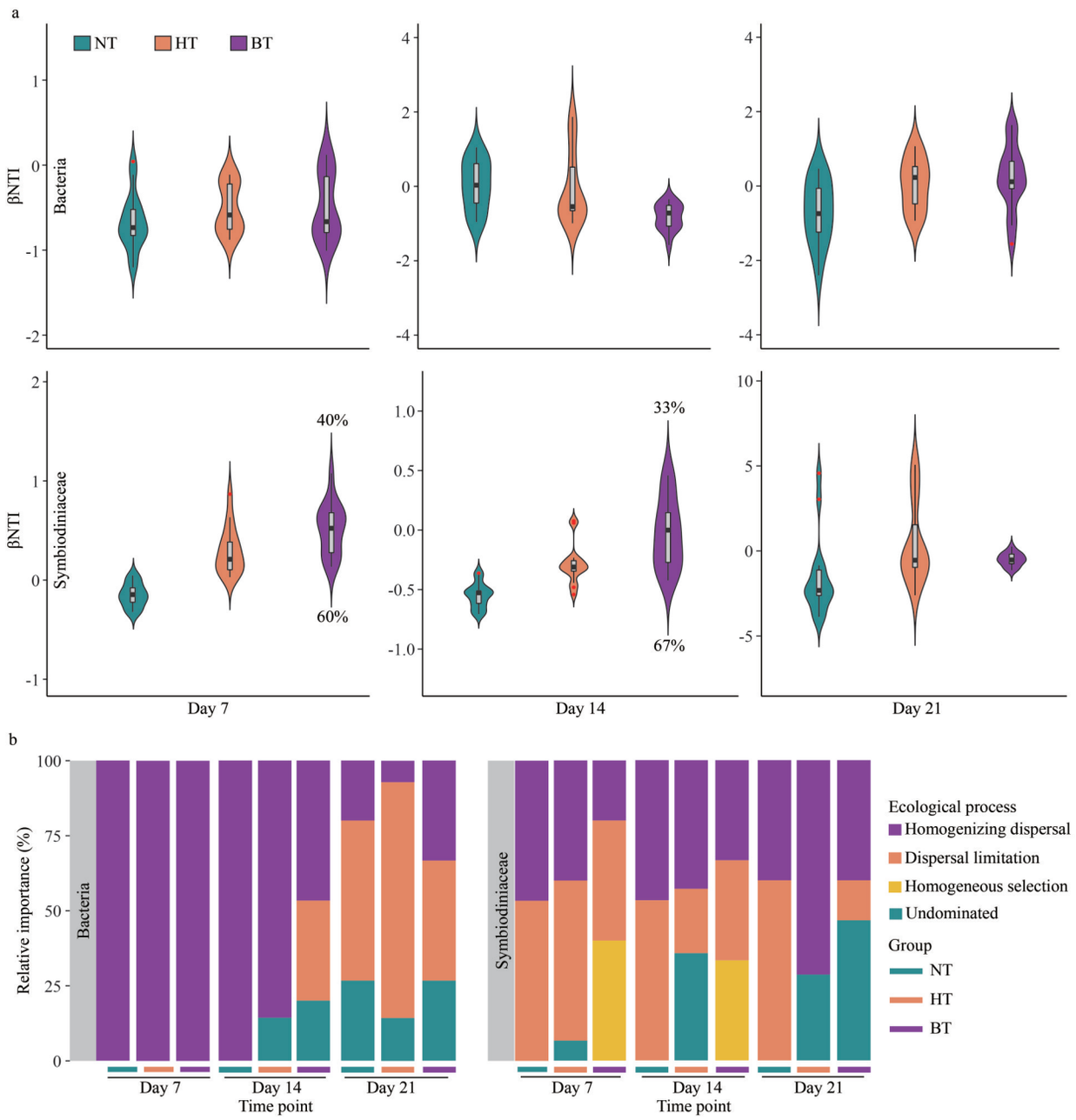

Fig.4 Stochastic and deterministic processes in bacterial and Symbiodiniaceae community assembly

a. relative contribution of stochastic and deterministic processes to the microbiome assembly among different groups based on the β-Nearest Taxon Index (βNTI) throughout the experiment. The βNTI values were calculated through null model analysis, with |βNTI|<2 and |βNTI|≥2 representing dominant stochastic and deterministic processes in microbiome assembly, respectively. The numbers below and above the violin plot indicate the percentage of stochastic processes and deterministic processes within microbiome assembly, respectively; b. relative importance of ecological processes including homogenizing dispersal (|βNTI|<2 and RC_{Bray}<-0.95), dispersal limitation (|βNTI|<2 and RC_{Bray}>0.95), homogeneous selection (βNTI>+2), and undominated (|βNTI|<2 and |RC_{Bray}|<0.95) based on βNTI and Bray-Curtis-based Raup-Crick Index (RC_{Bray}).

groups, *Thermobifida* abundances decreased. On Day 7, several genera, such as *Halodesulfovibrio*, *Ruegeria*, and *Shimia*, showed statistically different abundances between the NT and HT groups (Wilcoxon test, P<0.05) (Fig.5d). On Day 14, *Halarcobacter*, *Novibacillus*, *Endozoicomonas*, *Sphingobium*, and *Terribacillus* abundances were significantly lower in the HT group than in the NT group (Wilcoxon test, P<0.05). However, *Rhodococcus*, *Geobacillus*, and *Ralstonia* abundances were significantly higher in the HT group as compared with the BT group (Wilcoxon test, P<0.05). On Day

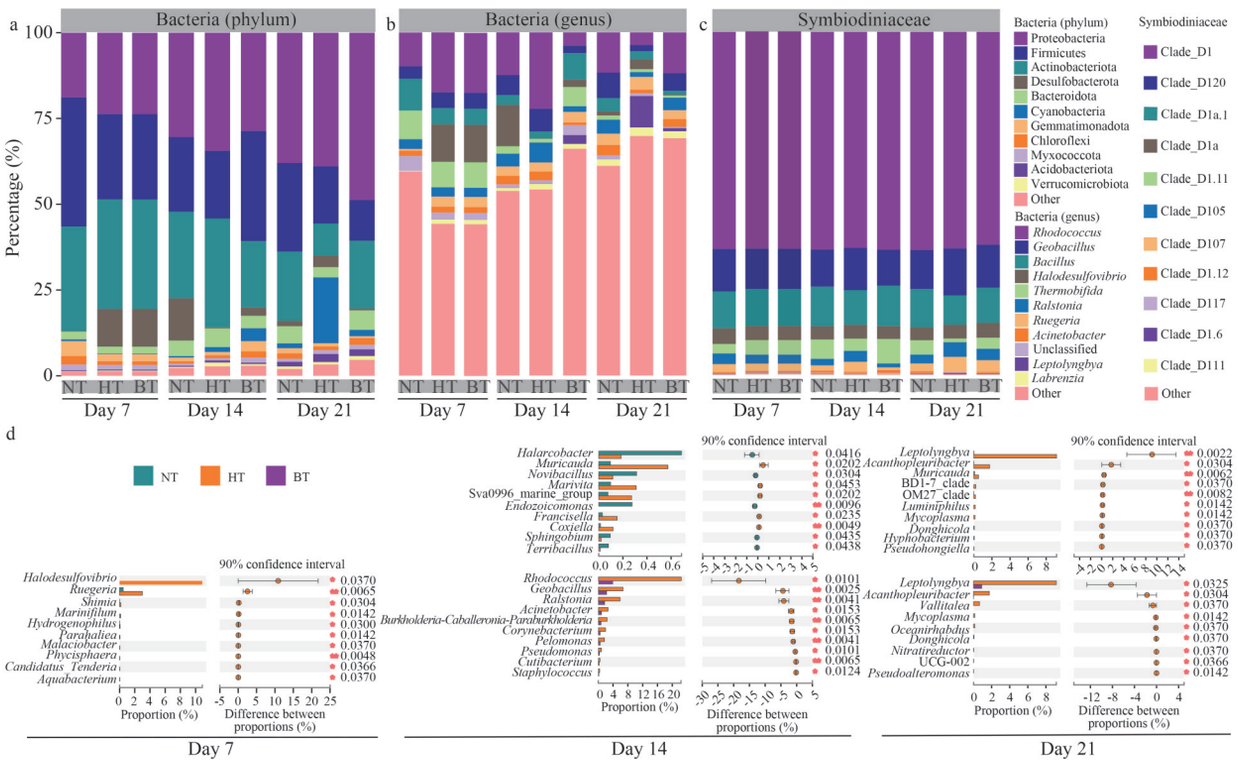

Fig.5 Temporal dynamics of bacterial and Symbiodiniaceae communities associated with corals of all experimental groups across three weeks

a. bacterial community composition at the phylum level; b. bacterial community composition at the genus level; c. changes of clade D Symbiodiniaceae; d. differences in bacterial community at the genus level across three weeks. Only the significantly different groups are shown. Genera or clades with a lower abundance than the top 12 in each group were classified as "other". The bacterial community within the NT group was compared to that of the HT group on Day 7, and the bacterial communities within the NT and BT groups were both compared to that of the HT group on Day 14 and Day 21, respectively (only the top 10 differential bacterial genera were listed).

21, the NT and BT groups had significantly lower abundances of *Acanthopleuribacter*, *Mycoplasma*, and *Donghicola* compared to the HT group (Wilcoxon test, $P<0.05$).

The dominant group of Symbiodiniaceae communities across all samples was subclade D1 of *Durusdinium* throughout the experiment (Fig.5c). The Symbiodiniaceae community composition presented no significant dissimilarity over 3 weeks of the experiment. At the ASV level, the ASVs specific to the HT group increased across 3 weeks (Supplementary Fig.S6). In addition, ASVs predominantly belonging to Firmicutes were depleted in the HT group on Day 14. In contrast, ASVs belonging to Proteobacteria were enriched in the BT group on Day 21.

Microbiomes from corals have tested positive for our inoculant strain FRS1. On Day 7 and Day 14, no sequences affiliated to the inoculum FRS1 was detected in the coral microbiome. On Day 21, FRS1 sequences were present in all three treatments, and BT had a higher abundance than NT or HT, but since each treatment had only one replicate, this was not statistically significant. Linear discriminant analysis of Effect Size (LEfSe) was used to detect ASVs with significant differences among all groups. Subsequently, a total of 40 ASVs were identified as potential biomarkers related to coral thermal stress (Fig.6). On Day 14, ASV59 belonging to the genus *Vulgatibacter* of the phylum Myxococcota was more abundant in the NT and BT groups than in the HT group. ASV1372 assigned to the family Thermomicrobiaceae was enriched in the NT and BT groups and depleted in the HT group throughout the entire experiment. Conversely, the abundance of ASV86 assigned to the order Gaiellales was higher in the HT group compared with that in the NT and BT groups throughout the whole experiment. ASV3655, classified as *Endozoicomonas*—a genus that is abundant in coral holobionts across the globe, decreased in the HT and BT groups. Four ASVs belonging to the family Rhodobacteraceae were identified as biomarkers. Of these, the abundances of ASV113, ASV34, and ASV961 increased in the

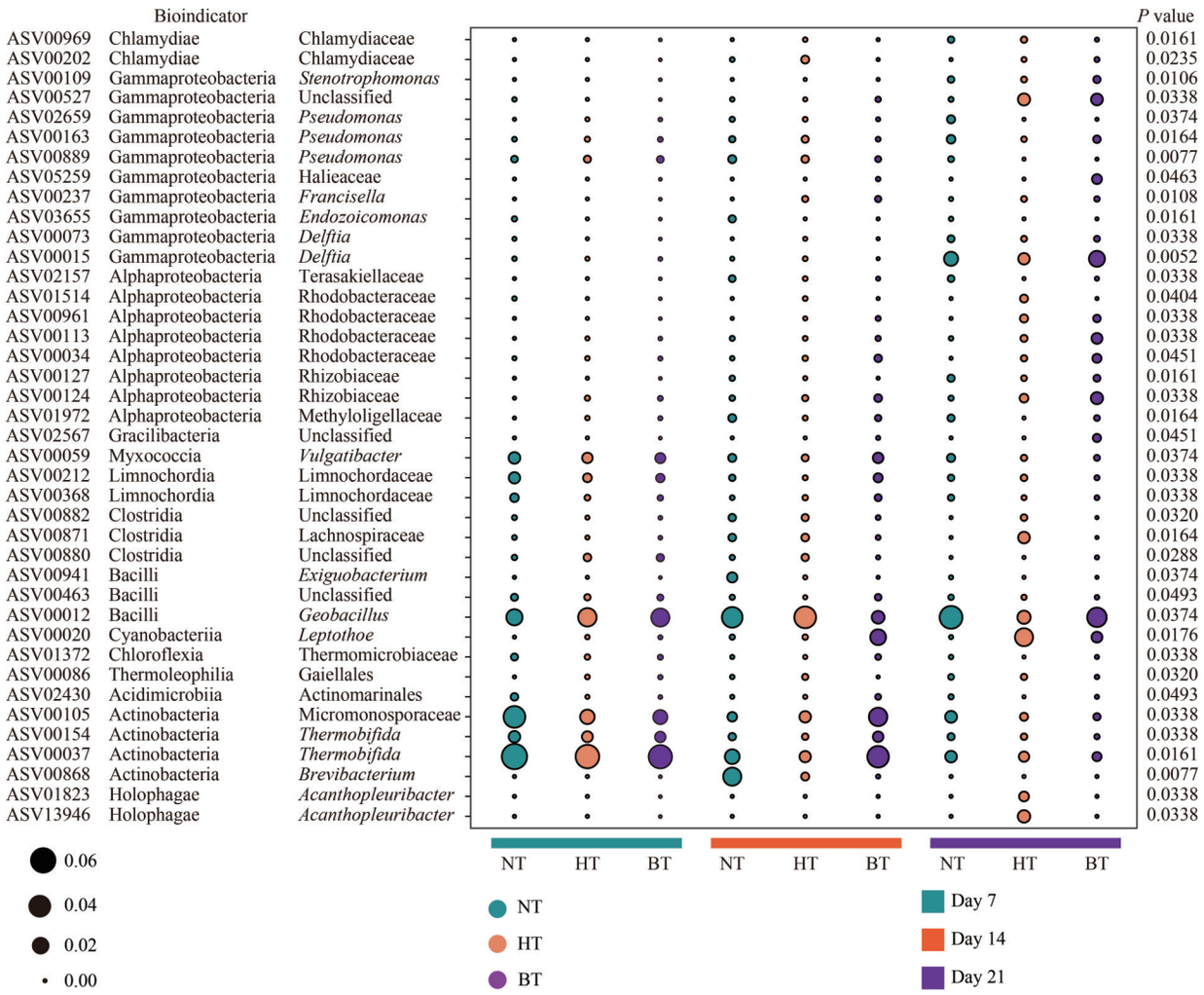

Fig.6 Bubble graph showing relative abundance of biomarker amplicon sequence variants (ASVs) in each experimental group across 3 weeks

The relative abundance is shown below the graph for all biomarker ASVs. Bubbles with statistically significant ASVs (P<0.05), as determined by the nonparametric Kruskal-Wallis test, are shown.

HT and BT groups during thermal stress. However, the abundance of ASV1514 only increased in the HT group.

3.5 Temporal dynamic of bacteria and Symbiodiniaceae co-occurrence networks

Co-occurrence network analysis was performed to analyze the effect of the addition of ROS-scavenging bacteria on bacteria-Symbiodiniaceae interactions across the 3 weeks of the experiment. In the NT group, the interactions between bacteria and Symbiodiniaceae were similar across the 3 weeks, and there was no significant difference in the degrees of bacteria and Symbiodiniaceae (Fig.7; Supplementary Fig.S7). In the HT group, the interactions between bacteria and Symbiodiniaceae weakened during the experiment process; furthermore, the degree of Symbiodiniaceae was significantly higher than that of bacteria on Day 14, which indicated that Symbiodiniaceae played a more important role than bacteria in the co-occurrence network. After the addition of the ROS-scavenging bacteria, the interactions between bacteria and Symbiodiniaceae in the BT group became more complex, showing a higher degree of bacteria compared with Symbiodiniaceae. The “network hubs” were further defined based on nodes with high values of degree (≥10) and closeness centrality (>0.3) in the co-occurrence network, and this revealed that the number of network hubs varied among different groups (Supplementary Fig.S7). There were 25 network hubs on Day 7, 15 on Day

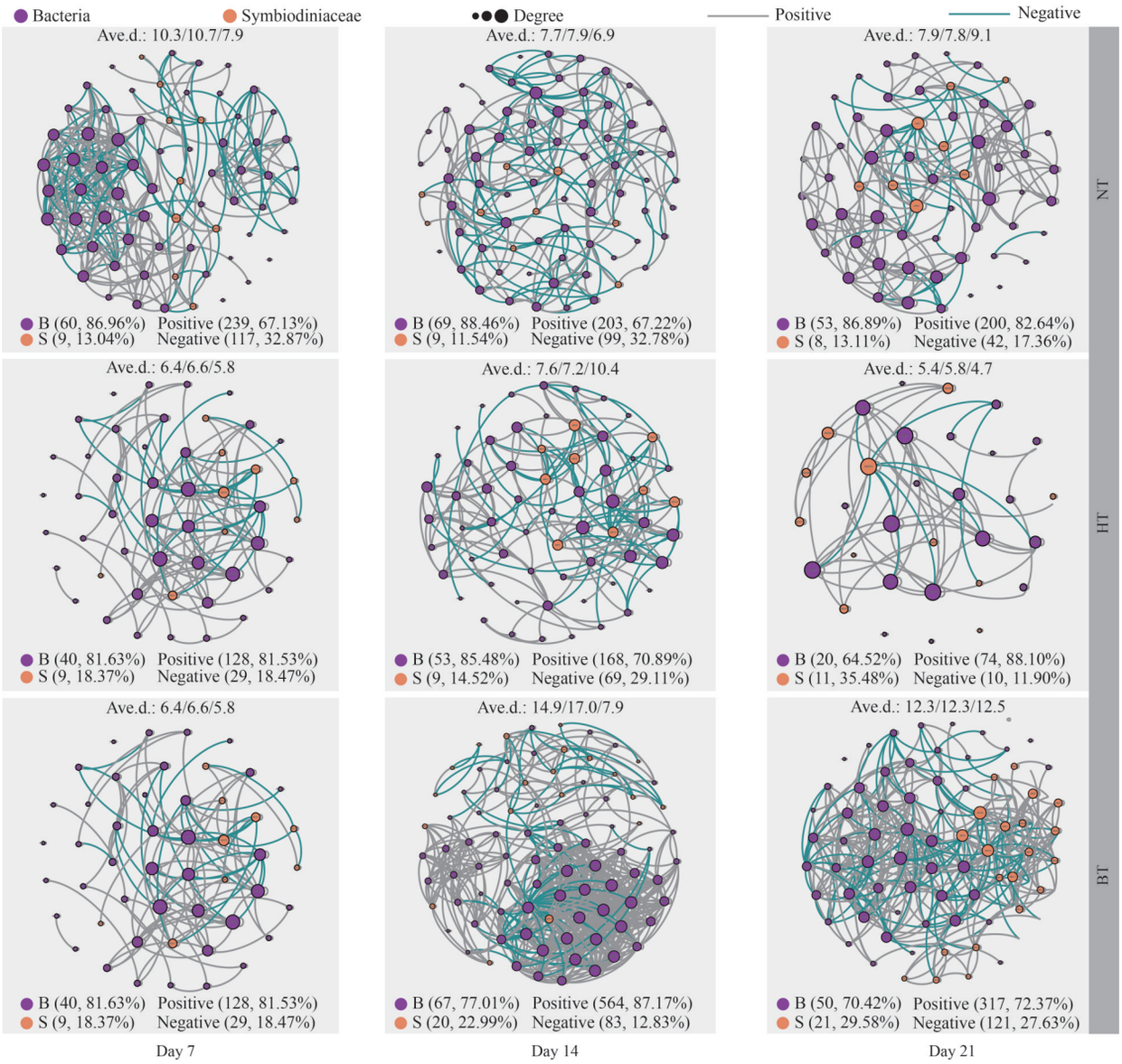

Fig.7 Temporal dynamics of bacteria-Symbiodiniaceae interkingdom networks of each group

Co-occurrence network analysis of microbial communities associated with all coral samples showed microbial interkingdom network patterns differed among NT, HT, and BT groups at different stages of the experiment process. Ave.d: average degree of whole taxa/Bacteria taxa/symbiodiniaceae taxa.

14, and 17 on Day 21 for the NT group (Supplementary Fig.S7). The HT group had 10 network hubs on Day 7, 18 on Day 14, and 5 on Day 21. As the number of network hubs increased rapidly after adding ROS-scavenging bacteria, 48 and 44 network hubs were identified in the BT group on Day 14 and on 21, respectively.

For the network of the bacterial community, the interaction patterns of bacteria differed among the three experimental groups (Supplementary Fig.S8). In the NT group, the average degree of the bacterial network weakened during the experiment (Supplementary Table S3), while in the HT group, the average degree of the bacterial network increased slightly on Day 14 but then decreased severely on Day 21. In contrast, the average degree of the bacterial network in the BT group became significantly higher on Day 14 and was higher than that of the HT group on Day 21.

4 DISCUSSION

Coral reefs are declining rapidly due to both global and local disturbances, such as large-scale coral bleaching and death caused by rising ocean surface temperature (Hughes et al., 2017). Thermal

stress can destabilize the nutrient cycling between corals and the associated Symbiodiniaceae, which leads to the breakdown of coral-algal symbiosis (Rädecker et al., 2021). Moreover, heat stress induces the excessive production of intracellular ROS by coral holobionts (Lesser, 2011; Levin et al., 2016). If not scavenged, excess ROS may trigger a signaling cascade that culminates in the dissociation of the coral host and algal symbiont in a process known as bleaching. Bacteria can directly affect the concentration of ROS in many organisms as an important sink (Morris et al., 2022). Microbes with the capacity of free radical removal may strengthen the resilience of corals to rising temperatures by scavenging more ROS to reduce the damage caused by these molecules (Dungan et al., 2021). The current study explored the potential beneficial effects of the addition of ROS-scavenging bacteria on the thermal stress resilience of corals.

4.1 Effect of the addition of ROS-scavenging bacteria on the physiological state of corals

In the present study, thermal stress caused bleaching of the corals without added bacteria, while the corals inoculated with ROS-scavenging bacteria were visibly healthy. Heat stress damages the temperature-related photosystem II electron transport of the Symbiodiniaceae, and decreases photosystem II photochemical efficiency (F_v/F_m) (Takahashi et al., 2007). Consequently, the symbiotic relationship of corals and Symbiodiniaceae and its density may be affected (Amid et al., 2018). In the current study, physiological indexes including photosystem II photochemical efficiency, protein content, and Symbiodiniaceae density in corals inoculated with ROS-scavenging bacteria were higher compared with those of corals without bacteria addition across 3 weeks. Moreover, the changing profiles of protein content within corals among the three experimental groups resembled patterns of Symbiodiniaceae density. This similarity is congruent with a study that demonstrated the presence of proportional relationships between Symbiodiniaceae densities and coral tissue biomass (Kochman et al., 2021).

Feeding was previously shown to enhance corals' photosynthetic efficiency, Symbiodiniaceae density, and protein content (Houlbrèque et al., 2004). The density of FRS1 probiotics applied was 1×10^5 cells/mL, which is significantly lower than the bacterial density found in natural reef waters and should be negligible from a nutritional point of view (Gast et al., 1998; Garcias-Bonet et al., 2023). This assumption is reinforced by our results, indicating that the protein contents of BT samples did not increase significantly compared with the NT samples without FRS1 inoculation.

ROS release of thermal-stressed corals inoculated with ROS-scavenging bacteria was lower than that of thermal-stressed and uninoculated corals. A better coral physiological status was consistent with the decreased release of ROS in corals inoculated with ROS-scavenging bacteria. This indicates that ROS-scavenging bacteria are involved in the removal of ROS. Different studies have demonstrated that the customized use of probiotics (Peixoto et al., 2019) can mitigate the effects of thermal bleaching, disease, oil spills and even promote coral calcification and growth (Fragoso et al., 2015; Rosado et al., 2019; Santoro et al., 2021; Zhang et al., 2021b; Li et al., 2023; Moradi et al., 2023; Ushijima et al., 2023). Based on these findings, we considered that ROS-scavenging bacteria could promote coral health including higher photochemical efficiency, protein content, Symbiodiniaceae density and chlorophyll-*a* content, and could also improve tolerance of *P. damicornis* to thermal stress and mitigate the effects of thermal stress on *P. damicornis* through scavenging ROS. More studies are necessary to clarify the underlying mechanisms involved in the observed health improvements in the future.

The increase of antioxidants in the coral host in response to heat stress occurred before the algal symbionts and prior to the photo-physiological decline, indicating that the photosynthetic pressure may be a late-stage response in bleaching (Hawkins et al., 2015; Krueger et al., 2015). However, the initial increase in antioxidants in coral hosts suggested the role of ROS in the early bleaching stage, which needs to be further investigated. According to Nielsen et al. (2018), however, ROS levels in endosymbionts could not be linked to physiological damage in coral hosts or endosymbionts, suggesting oxidative stress may not be the cause of coral bleaching. Moreover, in heat-stressed coral cells, the correlation between host and endosymbiont ROS may be due to metabolic stress in both partners or to a diffusive transfer of ROS. As a result, coral bleaching may occur through alternative mechanisms. Previously, it has been shown that a disruption of symbiotic nutrient cycling resulted in host starvation under heat stress days before any bleaching symptoms became

apparent (Rädecker et al., 2021). As such, future studies should emphasize to disentangle potential interactions of oxidative stress (through ROS overproduction) with disturbed symbiotic nutrient cycling in corals.

4.2 Effect of the addition of ROS-scavenging bacteria on coral-associated bacterial and Symbiodiniaceae community composition

There is evidence that the diversity of coral-associated microbial communities increases when corals are disturbed by environmental stressors (McDevitt-Irwin et al., 2017, 2019). Correspondingly, bacterial alpha diversity in the HT and BT groups increased significantly under thermal stress in the current study. Corals naturally change their bacterial community composition and may uptake microbes from the surrounding environment (Ziegler et al., 2019; Villela, 2020; Doering et al., 2021). The addition of ROS-scavenging bacteria promoted shifts in the coral microbiome, and the consistency of alpha-diversity changes in the BT group might indicate the effect of the inoculant. Additionally, the "Anna Karenina principle" for animal microbiomes showed that there was greater variation in the microbial community composition of dysbiotic individuals compared with healthy individuals (Zaneveld et al., 2017). The stability in the alpha diversity of the NT group and the variability in the alpha diversity of the HT group in the current study were both congruent with the "Anna Karenina principle" (Zaneveld et al., 2017). In addition, bacterial inoculation may account for the high variability in the BT group.

Restructuring of a coral microbiome can occur on a relatively short timescale, and the duration of the probiotic effect following the addition of bacteria is not yet known (Rosado et al., 2019; Doering et al., 2021; Zhang et al., 2021b). In the current study, microbiomes from corals have tested positive for our inoculant strain FRS1. On Day 7 and Day 14, no FRS1 sequence was detected in the coral microbiome. On Day 21, FRS1 sequences were present in all three treatments, and BT had a higher abundance than NT or HT, but since each treatment had only one replicate, which was not statistically significant (Supplementary Table S4) and suggested that probiotics do not need to colonize the host to be effective as they can trigger specific responses and microbiome restructuring even without colonization (Garcias-Bonet et al., 2023). There was a significant difference in the beta diversity of the bacterial communities of the HT and BT groups on Day 14; however, this difference disappeared by Day 21, indicating that the effect of bacteria addition on the beta diversity of the BT group was not sustained to the end of the experiment. Previous studies have shown that the microbiome tends to return to its original state after probiotic inoculation ceases (Santoro et al., 2021). Moreover, there was also a significant difference in beta diversity between the NT and HT groups on Day 21. This supports previous evidence that thermal stress has a strong effect on the beta diversity of the coral microbiome (Maher et al., 2020). In addition, the current study demonstrated that beta-diversity dissimilarity of bacterial communities was predominantly explained by species turnover, and the proportion of species nestedness decreased across 3 weeks. Moreover, the percentage of dispersal limitation increased in the bacterial communities of all groups on Day 21. These findings of species turnover and dispersal limitation were in accordance with a previous report that microbial species turnover may be caused by dispersal limitation (Marcelino et al., 2018). Homogeneous and heterogeneous selection were recently reported to contribute greatly to the assembly of the coral microbiome (Zhang et al., 2021a); however, only the ecological process of homogeneous selection significantly contributed to the assembly of bacterial communities in the current study. This discrepancy could be attributed to the differences between the indoor culture environments in the current study and the more complex environmental factors in nature.

The composition pattern of coral-associated bacterial communities is essential for coral health and is sensitive to environmental stressors (Epstein et al., 2019). In the present study, the relative abundances of several bacterial taxa were significantly different after the addition of ROS-scavenging bacteria. In particular, the relative abundance of the phylum Myxococcota increased significantly after the addition of bacteria. Myxococcota is usually enriched in heat-tolerant corals and contributes to coral resilience under heat stress (Ziegler et al., 2017; Li et al., 2021). The high abundance of Myxococcota in the current study indicates that the addition of ROS-scavenging bacteria might mitigate bleaching by favoring the proliferation of thermally tolerant bacteria (Boilard et al., 2020). Members of the Actinobacteria, such as the genus *Micromonospora*, were proposed to contribute to

coral health (Kuang et al., 2015). This study found that *Micromonospora* ASVs were detected in the BT group but not in the HT group. This indicates that adding ROS-scavenging bacteria increases the abundance of beneficial bacteria. Bacteria enriched in the NT group, such as Chloroflexi, were also enriched in the BT group after inoculation with ROS-scavenging bacteria. However, bacteria enriched in the HT group were specific, with none of them enriched in the NT and BT groups. This similarity in the bacterial community structures of the NT and BT groups might therefore have contributed to their relatively close physicochemical statuses.

Microbial interactions are intrinsic factors that are potentially primary drivers of coral microbial community structure and holobiont homeostasis, which are affected by high temperatures to different extents (Lima et al., 2020). In the current study, interactions among different bacterial taxa of thermal-stressed corals were more complex compared with those of normal corals on Day 14. Recent studies revealed that long-term warming significantly strengthened the complexity and stability of networks in grassland soil microbial communities (Yuan et al., 2021). In contrast, findings from the current study showed that the network complexity of the bacterial community of the HT group was simplified largely on Day 21. This difference may be due to thermal stress exceeding the buffering capacity of microbial partners, which leads to coral death (Epstein et al., 2019; Boilard et al., 2020). Microbial alpha diversity is intimately relevant to co-occurrence network complexity (Chen et al., 2021). In the present study, higher alpha diversity is consistent with the more complex network of the BT group. Despite being under thermal stress, a higher complexity of network in the BT group may be indicative of the effects of the inoculant. Based on these findings, we speculate that the reinforced complexity of a microbial network after bacteria addition may promote coral health under thermal stress. This is different from the complexity resulting solely from heat disturbance.

In the current study, *Durusdinium* subclade D1 dominated the Symbiodiniaceae community in agreement with a previous study on *Pocillopora damicornis* (Jandang et al., 2022). In addition, the absence of significant differences in the alpha diversity, beta diversity, and composition of the Symbiodiniaceae community might support previous reports that there were no changes in the Symbiodiniaceae community composition of corals resistant to thermal stress (Bellantuono et al., 2012; Epstein et al., 2019). Microbiological changes induced by many perturbations are stochastic (Villela, 2020), thus the involvement of a deterministic process in the assembly of the Symbiodiniaceae community might indicate the effect of bacteria addition.

4.3 Effects of ROS-scavenging bacteria addition on the co-occurrence of coral-associated bacteria and Symbiodiniaceae

Both bacteria and Symbiodiniaceae are vital for the biological functions of corals (Dungan et al., 2021; Pogoreutz et al., 2022; Tandon et al., 2022). The structures of both bacterial and Symbiodiniaceae communities become more complex when corals are under thermal stress, with indicators including higher alpha- and beta-diversity (Pootakham et al., 2019; Claar et al., 2020). Matthews et al. (2020) proposed that the bacteria-Symbiodiniaceae-coral relationship underpins the nutrition and stress tolerance of a coral holobiont. In the current study, the interactions between bacteria and Symbiodiniaceae in coral samples became more intimate after bacteria addition. This may indicate that the inoculant promotes coral health by regulating the interactions between bacteria and Symbiodiniaceae. Bacterial inoculation of corals resulted in changes to the bacterial network and the co-occurrence network of bacterial and Symbiodiniaceae taxa. In accordance with pathogen invasion strengthening the interdomain network complexity of bacterial and fungal taxa (Tan et al., 2021), the corals inoculated with ROS-scavenging bacteria in the current study had a more complex co-occurrence network of bacterial and Symbiodiniaceae taxa. Notably, Symbiodiniaceae was relatively stable in the co-occurrence network of bacterial and Symbiodiniaceae taxa, the roles of both bacteria and Symbiodiniaceae in the network were strengthened after bacteria addition, and the interaction between bacteria and Symbiodiniaceae in corals of the HT group was markedly weakened after exposure to thermal stress for 2 weeks. Therefore, the addition of ROS-scavenging bacteria may promote coral health by strengthening the complexity of the co-occurrence network of bacterial and Symbiodiniaceae taxa.

5 CONCLUSION

Coral holobionts produce excess reactive oxygen

species (ROS) during thermal stress, which seems to be one of the triggers of coral bleaching. ROS-scavenging bacteria can neutralize excess ROS, and may therefore benefit corals in resisting heat stress. We explored the potential beneficial effects of ROS-scavenging bacteria on the resilience of corals to heat stress. The addition of ROS-scavenging bacteria enhanced coral health, reduced ROS concentration, changed coral-associated microbiomes, and strengthened the interaction between coral-associated bacteria and Symbiodiniaceae. These findings suggest that ROS-scavenging bacteria have potential beneficial effects on thermal stress coral *Pocillopora damicornis* and provide a reference for the manipulation of microbes to restore coral reefs in the future.

6 DATA AVAILABILITY STATEMENT

The datasets presented in the current study have been deposited in the NCBI Sequence Read Archive database, with BioProject IDs of PRJNA820977 and PRJNA821388.

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Electronic supplementary material

Supplementary material (Supplementary Tables S1–S4 and Figs.S1–S8) is available in the online version of this article at https://doi.org/10.1007/s00343-024-3159-0.