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Deciphering the disturbance mechanism of BaP on the symbiosis of *Montipora digitata* via 4D-Proteomics approach

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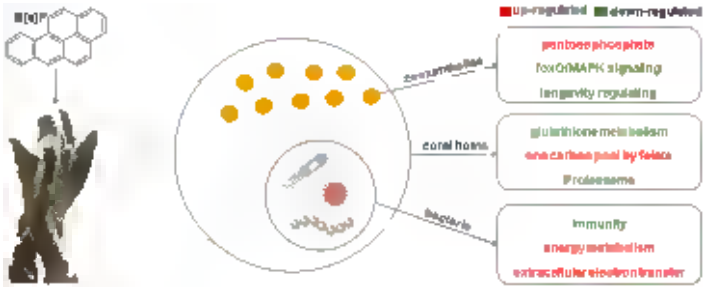
HIGHLIGHTS

- Differentially expressed proteins of coral were identified using the diaPASEF proteomics approach.
- Assessing the effects of B [a]P on corals from the perspective of symbionts.
- B [a]P disrupts the antioxidant and protein degradation capacity of coral polyps.
- B [a]P induced apoptosis in zooxanthellae by inhibiting the expression of SOD2-related proteins.
- Coral symbiotic microorganisms adapt to the stress of B [a]P by enhancing extracellular electron transport.

GRAPHICAL ABSTRACT



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ABSTRACT

The coral holobiont is mainly composed of coral polyps, zooxanthellae, and coral symbiotic microorganisms, which form the basis of coral reef ecosystems. In recent years, the severe degradation of coral reefs caused by climate warming and environmental pollution has aroused widespread concern. Benzo(a)pyrene (BaP) is a widely distributed pollutant in the environment. However, the underlying mechanisms of coral symbiosis destruction due to the stress of BaP are not well understood. In this study, diaPASEF proteomics and *16S rRNA* amplicon pyrosequencing technology were used to reveal the effects of 50 μg/L BaP on *Montipora digitate*. Data analysis was performed from the perspective of the main symbionts of *M. digitata* (coral polyps, zooxanthellae, and coral symbiotic microorganisms). The results showed that BaP impaired cellular antioxidant capacity by disrupting the GSH/GSSG cycle, and sustained stress causes severe impairment of energy metabolism and protein degradation in coral polyps. In zooxanthellae, BaP downregulated the protein expression of SOD2 and mtHSP70, which then resulted in oxidative free radical accumulation and apoptosis. For coral symbiotic microorganisms, BaP altered the community structure of microorganisms and decreased immunity. Coral symbiotic microorganisms adapted to the stress of BaP by adjusting energy metabolism and enhancing extracellular electron transfer. BaP adversely affected the three main symbionts of *M. digitata* via different mechanisms. Decreased

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antioxidant capacity is a common cause of damages to coral polyps and zooxanthellae, whereas coral symbiotic microorganisms are able to appropriately adapt to oxidative stress. This study assessed the effects of BaP on corals from a symbiotic perspective, which is more comprehensive and reliable. At the same time, data from the study supports new directions for coral research and coral reef protection.

1. Introduction

The coral reef ecosystem is one of the most biologically diverse ecosystem on earth, with high ecological services and economic value (Gong et al., 2020a). The coral holobiont, mainly formed by the symbiosis of coral polyps, zooxanthellae, and coral symbiotic microorganisms, is the basis for the prosperity of coral reef ecosystems (Baumgarten et al., 2013; Richards and McCutcheon, 2019). Within the coral holobiont, coral polyps provide zooxanthellae and bacteria with lodging sites and metabolites, and zooxanthellae provide coral polyps with the energy (up to 90% of total energy needs) necessary for growth and development. Coral symbiotic microorganisms play an important role in coral immunity, nutrient supply, and adaptability (Shu et al., 2020; Maire and van Oppen, 2021). Adverse factors such as high temperatures, pollutants, and disease can disrupt the symbiotic system of corals and exacerbate coral bleaching or death (Claar et al., 2020). Therefore, it is important to explore the mechanism of adverse factors disturbing the symbiotic relationship for the protection of corals.

Over the last 30 years, many large-scale coral bleaching events have occurred worldwide, and resulted in a 50% reduction in coral coverage (Selmoni et al., 2020). Existing studies have shown that rising sea temperature is the main cause of the large-scale bleaching and death of corals (Hughes et al., 2017). In addition, environmental pollutants pose a serious threat to the survival of corals (Reichert et al., 2018; Marangoni et al., 2020). Polycyclic aromatic hydrocarbons (PAHs) are a class of toxic substances produced by incomplete combustion of tobacco, oil, natural gas, etc. that are widely distributed in nature (Han et al., 2022). BaP is a typical polycyclic aromatic hydrocarbon that has been widely studied as a PAH model due to its ecotoxic, carcinogenic, and mutagenic properties (Williams and Hubberstey, 2014; Liamin et al., 2018). At present, researchers have studied the influence and potential risks of BaP on humans, scallops, zebrafish, etc (Li et al., 2021a; Hawkey et al., 2022; Wu et al., 2022). In addition, the impact of BaP on corals and the potential mechanisms of action that result in coral bleaching and death have recently attracted attention. Investigations have shown that the concentration of BaP in corals around Hainan Island in China ranges from 1.13 ng/g to 42.50 ng/g (Xiang et al., 2018). In a study of adult corals, BaP caused oxidative damage to coral polyps and severely affected the health of zooxanthellae (Xiang et al., 2019). Although studies on the mechanism of BaP action on coral health have been conducted, most of them have focused on changes that occur at the transcriptome and the physiological level. However, in corals, relatively few changes in the transcriptome translate to changes of the proteome. Thus, data at the transcriptome level may not fully reflect true phenotypic changes in the coral (Mayfield et al., 2018), which appears justified for conducting proteomic investigation on corals under BaP stress.

Proteins execute physiological functions and are direct manifestations of life activity. Thus, changes in protein expression may more directly reflect changes in biological phenotypes (Zhang et al., 2020). In recent years, proteomics has been developed rapidly and been successfully used in coral research. Proteomics studies have shown that regeneration-related proteins in *Montastraea cavernosa* are associated with inflammation, the extracellular matrix, the skeleton, catabolism, and apoptosis (Horricks et al., 2020). With the help of proteomic analysis, researchers have found that the expression of proteins related to detoxification, antioxidation, and cell metabolism in offshore corals has increased, paralleling coral adaptability to human impact (Tisthammer et al., 2021). The wide application of proteomics has also promoted innovation in proteomics technology. In 2020, leading proteomics scientists Matthias Mann (Max Planck Institute of Biochemistry), Hannes Röst (University of Toronto), Ben Collins (ETH Zurich), Ruedi Aebersold (ETH Zurich) and their team members jointly developed the dia-PASEF (Meier et al., 2020). This technology is based on the timsTOF Pro platform and combines the advantages of data-independent acquisition (DIA) and parallel accumulation-serial fragmentation (PASEF) technology to comprehensively improve protein identification capabilities, detect sensitivity, and ensure data integrity. These properties of the timsTOF Pro platform allow for a broad application of proteomics. At present, the dia-PASEF proteomics technology has been successfully applied in the medical field (Fossati et al., 2021; Li et al., 2021b). However, this technology has not been reported for coral research.

Coral symbiotic microorganisms enhance the adaptation of corals to environmental changes and transient stress exposures (Bang et al., 2018; Maire and van Oppen, 2021). Coral symbiotic microorganisms also play a key role in coral nutrient metabolism and immunity (Modolon et al., 2020). *16S rRNA* amplicon pyrosequencing technology is widely used in the identification and analysis of coral symbiotic microorganisms. Recent studies have shown that healthy corals with associated bacteria have stronger resistance and antagonistic activity to stress than bleached corals, and coral symbiotic microorganisms may be used as an indicator of coral health (Patel et al., 2021). A comparative analysis of coral samples during different seasons showed that coral symbiotic microorganisms adapt to seasonal changes by adjusting their community structure and proportion (Yu et al., 2021). In addition to climate change, marine pollutants also affect coral symbiotic microorganisms. Both copper (65 μg Cu/L) and nickel (9050 μg Ni/L) cause coral bleaching after 36 h, but nickel almost does not alter the microbiome communities of corals (Gissi et al., 2019). In conclusion, although many studies on coral symbiotic microorganisms have been conducted, relevant studies on the effects of global organic pollutants like BaP on coral symbiotic microorganisms are lacking.

The mutualistic symbiosis of coral polyps, zooxanthellae, and coral symbiotic microorganisms allows corals to maintain high productivity in nutrient-poor tropical oceans (Sun et al., 2020). When coral symbiont encountered environmental changes, such as high temperature, pollutants stress, etc. the symbiotic relationship may breakdown, zooxanthellae escape from the coral host, and coral bleaching occurs (Ishii et al., 2018). Elevated temperature, marine pollutants, and bacterial infection are the main induce factors for the coral bleaching (Ben-Zvi et al., 2019). Global organic pollutants such as BaP cause coral bleaching. However, the mechanistic effect of each symbiont in this process is not clear. In this study, *M. digitata* was selected as the target species, which is a widespread species in the Indo-Pacific region, mainly in the South China Sea, the Philippines, Fiji, and the Great Barrier Reef of Australia, etc. The 4D-Proteomics approach was used to investigate the disturbance mechanism of BaP on the symbiotic relationship of *M. digitata* from the perspectives of coral polyps, zooxanthellae, and coral symbiotic microorganisms, which not only help us to better understand the response strategy of coral under the stress of marine pollutants, but also can provide the guidance for the protection of coral habitats.

2. Materials and methods

2.1. Acquisition and domestication of corals

M. digitata was collected from the coral breeding base in Wanning City, Hainan Province, China (110.5263°E, 18.9951°N). The coral

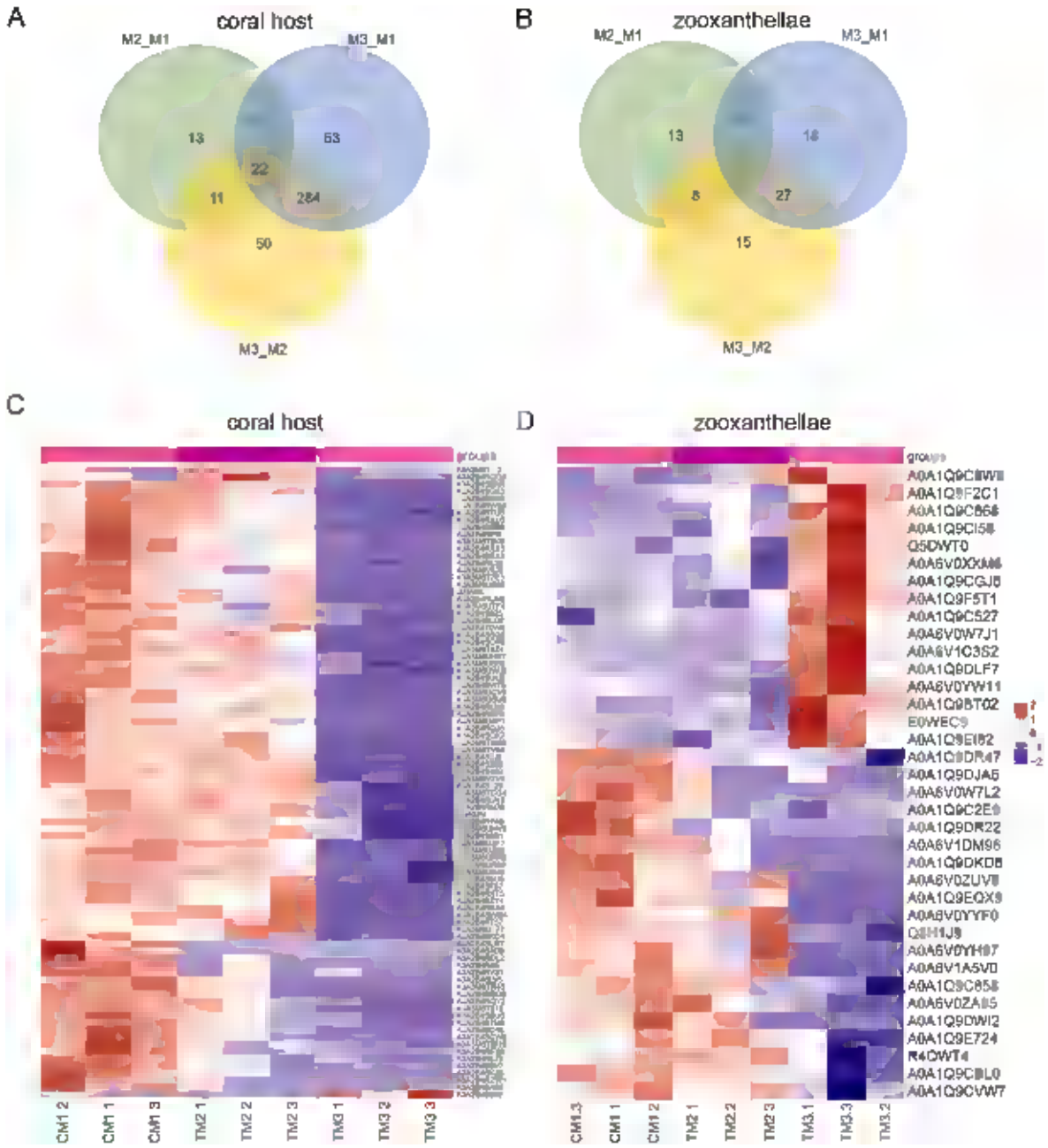

Fig. 1. Differentially expressed protein analysis of *Montipora digitata*. (A) Venn analysis of DEPs among the comparison groups of coral polyps. (B) Venn analysis of DEPs among the comparison groups of zooxanthellae. (C) Heatmap of coral polyps. (D) Heatmap of zooxanthellae.

samples were quickly transferred to the laboratory in Haikou City, Hainan Province once collected from the sea, and then divided into 6 × 10 cm (width × height) size branches, and each of them was fixed on a cement base. Fixed coral branches were immediately placed in a coral bionic tank in the animal breeding room for recovery and domestication. The coral domestication conditions were as follows: illumination intensity of 180 μmol photons/m^2/s^2; photoperiod of 12 h:12 h (light: dark); salinity, 35%; and temperature, 25 ± 0.2 °C. Domestication lasted for two months. After domestication, coral branches that were all in health condition were randomly selected for the experiment.

2.2. Experimental design

There were 2 study groups, i.e., the control and experimental groups, with 3 replicates per group. Treatment groups were kept in a 25 × 25 × 25 cm glass tank under the same basic conditions as those followed in the domestication tank. Half of the seawater was changed daily, followed by measurements of pH, temperature, and salinity. The experimental group was treated with a constant BaP concentration of 50 μg/L, whereas the control group was not treated with BaP. The treatment dosage was based on the results from preliminary experiments (Chen et al., 2021). The concentration for 50% of the maximal effect (EC_{50}) of BaP on *M. digitata* under 120 h–168 h stress was estimated as 47.00–104.10 μg/L. BaP powder (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in DMSO (Solarbio, Beijing, China). The experiment was conducted for 120 h in total, and sampling was performed at 0, 72, and 120 h. Samples from the control group, after 72 h treatment group, and 120 h treatment group were labelled CM1, TM2, and TM3, respectively, with three replicates of each group labelled after the decimal point of the group name. For example, the 3 replicates of the control group were

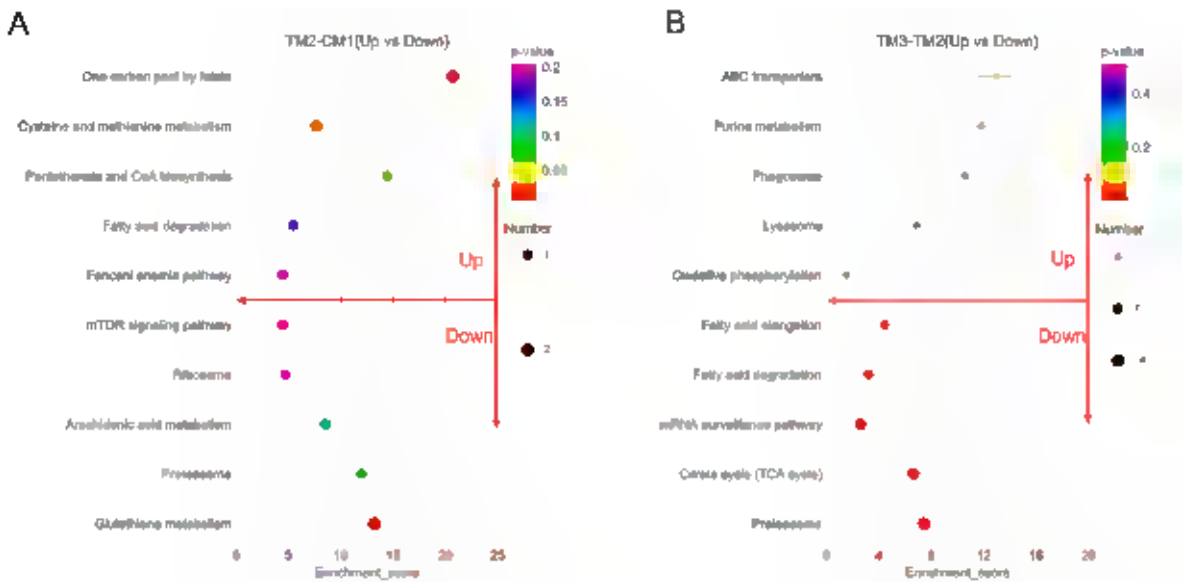

Fig. 2. KEGG analysis of DEPs in coral host. (A) KEGG analysis of DEPs in coral polyps in the early stage of BaP stress (TM2-CM1), the upper part is up-regulated, and the lower part is down-regulated. (B) KEGG analysis of DEPs in coral polyps in the late stage of BaP stress (TM3-TM2), the upper part is up-regulated, and the lower part is down-regulated.

labelled as CM1.1, CM1.2, and CM1.3. A portion of each sample was immediately used for zooxanthellae density statistical analysis, and other samples were snap frozen in liquid nitrogen and transferred to freezer at −80 °C for 4 h, later storage and further microbiome and proteome experiments.

2.3. Determination of zooxanthellae density and chlorophyll a content

Zooxanthellae density was used as an indicator of the number of symbiotic zooxanthellae, and chlorophyll *a* content was used to assess the physiological state of zooxanthellae. First, zooxanthellae were washed of 2 g fresh *M. digitata* tissue using a Waterpik-450EC dental scaler with sterile seawater. The liquid containing zooxanthellae was equally divided into two parts, one of which was used to calculate the density of zooxanthellae based on a previously described method (Qin et al., 2019). The other sample was used to measure the chlorophyll *a* of zooxanthellae by the method of Xiang et al. (2019).

2.4. diaPASEF proteomics evaluation and data analysis

2.4.1. diaPASEF proteomics evaluation

Compared with the traditional proteomics process, the diaPASEF process uses a more advanced timsTOF Pro mass spectrometry system, which has higher sensitivity and accuracy. The detailed process of protein group extraction and identification is shown in Supplementary Material Table S1.

2.4.2. Screening of differentially expressed proteins

Two standards were selected to calculate protein differences between samples. The fold change was used to evaluate differences in protein expression levels between treatment groups by the Student's *t*-test. The *p* value was used to determine significant differences between groups. Screening for protein expression differences was based on the following conditions: fold change = 1.2 and p value < 0.05, where FC = 0 and FC = inf, representing differences of "with or without."

2.4.3. Functional analysis of differentially expressed proteins

Several databases, such as Universal Protein (UniProt, https://www.UniProt.org/), Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.kegg.jp/), and Gene Ontology (GO, http://geneontology.org/), were used to extract protein annotation information and predict protein functions. The GO/KEGG function enrichment analysis was performed using the hypergeometric distribution test to calculate whether the representative function set was significantly enriched in the differentially expressed proteins (DEPs) list, and the *p* value was corrected using Benjamini & Hochberg's multiple tests to determine the false discovery rate (FDR).

2.5. High-throughput sequencing and analysis based on 16S rRNA

2.5.1. 16S rRNA sequencing

High-throughput sequencing technology was used to identify the symbiotic bacteria present in corals. In this study, the V3 and V4 variable regions of the bacterial *16S rRNA* gene were amplified and sequenced with the universal primers 343 F (5′-TACGGRAGGCAGCAG-3′) and 798R (5′-AGGGTATCTAATCCT-3′). Sequencing was performed on the Illumina MiSeq platform. 16S rRNA sequencing details are provided in the Supplementary Material Table S2.

2.5.2. PICRUSt analysis

The PICRUSt function predictive analysis was based on the *16S rRNA* sequencing data annotated using the Greengenes database (https://greengenes.lbl.gov/Download/). PICRUSt software was used to predict the composition of known microbial gene functions and to determine functional differences between different samples and groups. The Kruskal–Wallis algorithm was used to calculate differences between KEGG prediction results and generate a heatmap.

3. Results and discussion

3.1. Proteomic analysis

In this experiment, 733 coral host proteins and 310 zooxanthellae proteins were identified from *M. digitata* using diaPASEF (In the Supplementary Material, Tables S3 and S4). The protein concentration standard curve and SDS–PAGE results confirmed that the proteomic experiments were valid (in the Supplementary Material, Fig. S1A and C). A principal component analysis (PCA) showed that the 3 replicates of each experimental group were clustered together, which reflected the reproducibility of the experimental data (in the Supplementary

Table 1
Highlighted differentially expressed proteins in coral host under BaP stress.

Protein ID	Protein (gene symbol)	Fold change	DEP	Stage
A0A2B4RK22	SHMT2	2.716	up	TM2-CM1
A0A3M6TI13	ALDH1L	1.864	up	TM2-CM1
A0A2B4RL72	G6PD	0.551	down	TM2-CM1
A0A3M6T9K8	PRDX6	0.690	down	TM2-CM1
A0A3M6TXZ7	PSMB3	0.362	down	TM3-TM2
A0A2B4RQH9	MDH2	0.146	down	TM3-TM2
A0A2B4RTH4	PCK1	0.292	down	TM3-TM2
A0A2B4RS68	PCK2	0.140	down	TM3-TM2

Material, Fig. S1B and D). The Venn diagram shows the number of DEPs between groups (Fig. 1A and B), and the cluster heatmap displays the DEPs of each group (Fig. 1C and D). Overall, few differences in protein expression were found between CM1 and TM2, whereas many proteins were differentially expressed in TM3 compared to CM1.

3.2. Disruption effects of BaP on coral polyps

In the early stage (CM1-TM2) of BaP stress, 36 proteins were upregulated, and 23 were downregulated in the coral polyps. In the later stages (TM2-TM3) of BaP stress, 13 proteins were upregulated, and 354 proteins were downregulated. GO and KEGG databases were used for enrichment analysis of these DEPs.

GO analysis showed that in the early stage of BaP stress, the top 2 biological process (BP) terms were translation and methylation; the top 3 cellular component (CC) terms were membrane, extracellular region, and nucleus; and the top 3 molecular function (MF) terms were ATP binding, GTP binding and GTPase activity (in the Supplementary Material, Fig. S2A). In the later stages of BaP stress, the top 3 BP terms were translation, protein folding and intracellular protein transport; the top 3 CC terms were ribosome, proteasome complex, and spliceosomal complex; and the top 3 MF terms were ATP binding, RNA binding, and GTP binding (in the Supplementary Material, Fig. S2B). KEGG analysis revealed that in the early stage of BaP stress, the one carbon pool by the folate pathway was significantly upregulated, and the glutathione metabolism pathway was significantly downregulated (Fig. 2A). In the late stage, ABC transporter pathways were upregulated, while proteasome and tricarboxylic acid (TCA cycle) pathways were significantly downregulated (Fig. 2B). The DEPs used in KEGG analysis are shown in Table 1.

The one carbon pool by the folate pathway plays an important role in one-carbon metabolism (Moon et al., 2017). The upregulation of formyltetrahydrofolate dehydrogenase (ALDH1 L) promotes the conversion of 10-formyltetrahydrofolate (10-formyl-THF) to tetrahydrofolate (THF), and the upregulation of serine hydroxymethyltransferase (SHMT) promotes the interconversion of TFH and 5,10-methyl-TFH (Xie et al., 2013; Ruszkowski et al., 2019). 5,10-methyl-TFH is an intermediate metabolite within the metabolism of one carbon pool by folate and plays an important role in the one-carbon cycle (Rodriguez et al., 2019). The upregulation of ALDH1 L and SHMT promotes one-carbon metabolism in coral host cells.

Glutathione (GSH) is an important antioxidant that maintains intracellular redox balance and resists oxidative stress caused by foreign toxins (Tsugawa et al., 2019). Downregulation of glucose-6-phosphate 1-dehydrogenase (G6PD) and peroxiredoxin 6 (PRDX6) expression results in significant downregulation of the glutathione metabolism pathway. G6PD reduces NADP + to NADPH, which plays an important role in the glutathione/glutathione disulfide (GSSG) cycle (Swastika et al., 2020). PRDX6 belongs to the peroxiredoxin family and uses GSH to complete its catalytic peroxidatic reaction and generate GSSG (Arevalo and Vázquez-Medina, 2018). The downregulation of G6PD and PRDX6 expression disrupts the balance of GSH/GSSG and reduces the antioxidant capacity of coral host cells.

The proteasome is a protein complex responsible for the degradation of damaged or dysfunctional proteins (Hsu et al., 2020). In TM3, 26S proteasome regulatory subunits N3, N6, N9, 26S proteasome regulatory subunits N1, N2, T1, T2, T4, T6, and 20S proteasome subunits α2, α5, α6, β4 were not expressed, indicating that the structure and function of the proteasome were probably disrupted. Dysfunction of the proteasome disrupts intracellular protein turnover and the degradation of misfolded and damaged proteins, which leads to cellular proteotoxicity (Guo et al., 2020). The loss of multiple functional subunits of the proteasome caused by BaP results in the accumulation of misfolded and damaged proteins in coral host cells. These misfolded and damaged proteins are toxic to the host (Oakley et al., 2017).

The TCA cycle is a major source of cellular energy and participates in various metabolic pathways in cells (Kang et al., 2021). In TM3, malate dehydrogenase (MDH1), phosphoenolpyruvate carboxykinase (PCK),

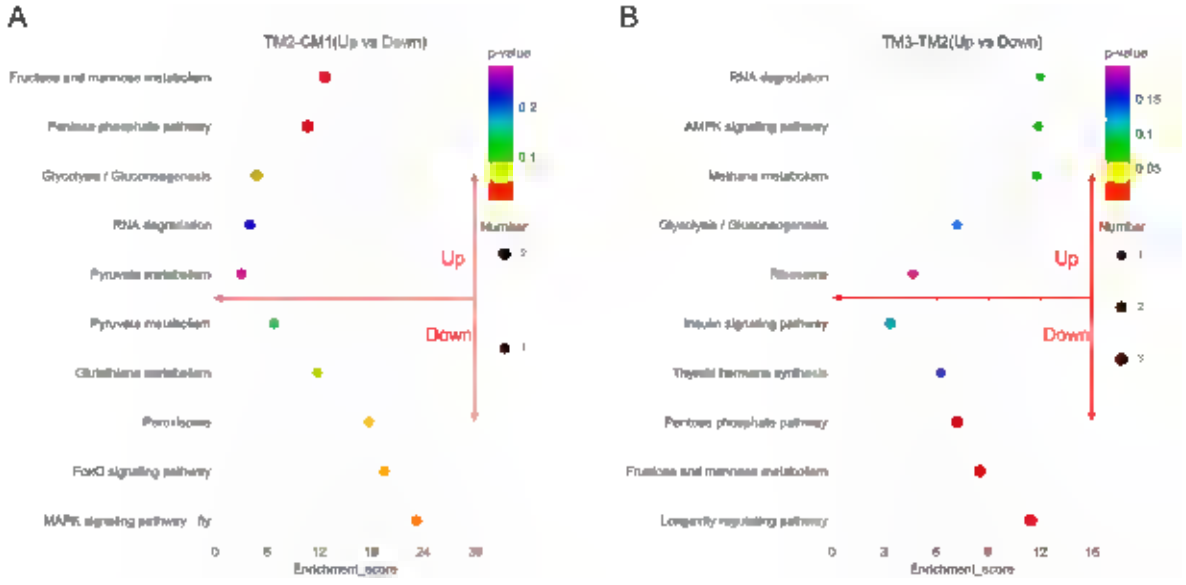

Fig. 3. KEGG analysis of DEPs in zooxanthellae. (A) KEGG analysis of DEPs in zooxanthellae in the early stage of BaP stress (TM2-CM1), the upper part is upregulated, and the lower part is down-regulated. (B) KEGG analysis of DEPs in zooxanthellae in the late stage of BaP stress (TM3-TM2), the upper part is upregulated, and the lower part is down-regulated.

Table 2
Highlighted differentially expressed proteins in zooxanthellae under BaP stress.

Protein ID	Protein (gene symbol)	Fold change	DEP	Stage
A0A1Q9CYG9	FBP	inf	up	TM2-CM1
A0A6V0WGX6	ALDO	inf	up	TM2-CM1
A0A1Q9D0S8	SOD2	0.596	down	TM2-CM1
A0A1Q9CJK1	mtHSP70	0.459	down	TM3-TM2
A0A1Q9DYJ8	mtHSP70	0.226	down	TM3-TM2
A0A6V1AH42	mtHSP70	0.0	down	TM3-TM2

fumarate hydratase (FH), citrate synthase (CS), ATP citrate (pro-S)-lyase (ACLY), dihydrolipoamide dehydrogenase (DLD), pyruvate dehydrogenase E1 component alpha subunit (PDHA), pyruvate carboxylase (PC), aconitate hydratase (ACO), and isocitrate dehydrogenase (IDH1, IDH3) were all downregulated. These proteins and enzymes are associated with multiple key reactions in the TCA cycle, and their absence or downregulation severely disrupts energy metabolism in coral polyps.

In conclusion, from CM1 to TM2, BaP disrupts the balance of GSH/GSSG and reduces the antioxidant capacity of coral polyps. At the same time, BaP increases intracellular one-carbon metabolism by enhancing THF cycling. From TM2 to TM3, BaP causes proteasome dysfunction and disrupts the TCA cycle network, which resulted in the inability to eliminate harmful proteins in coral host cells and disordered energy metabolism. These adverse factors contribute to the further deterioration or death of coral polyps.

3.3. Destruction effects of BaP on zooxanthellae

In the early stages (CM1-TM2) of BaP stress, 15 proteins were upregulated, and 7 were downregulated in zooxanthellae obtained from coral polyps. In the later stage (TM2-TM3) of BaP stress, 5 proteins were upregulated, and 46 proteins were downregulated. GO and KEGG databases were used for enrichment analysis of these DEPs.

GO analysis showed that in the early stage of BaP stress in zooxanthellae, the top 3 BP terms were malate metabolic process, carbohydrate metabolic process, and tricarboxylic acid cycle; the CC term was ribosome; and the top 3 MF terms were ATP binding, metal ion binding, and L-malate dehydrogenase activity (in the Supplementary Material, Fig. S3A). In the latter stage of BaP stress, the top 3 BP terms in zooxanthellae were photosynthesis, translation, and protein folding; the top 3 CC terms were ribosome, thylakoid membrane, and plasma membrane; and the top 3 MF terms were ATP binding, metal ion binding, and FMN binding (in the Supplementary Material, Fig. S3B).

KEGG analysis showed that in the early stage of BaP stress, fructose and mannose metabolism and pentose phosphate pathways were significantly upregulated, while Forkhead Box O (*FoxO*) and Mitogen-Activated Protein Kinase (MAPK) signalling pathways were significantly downregulated (Fig. 3A). In the late stage, the RNA degradation pathway was significantly upregulated, while fructose and mannose metabolism, pentose phosphate and longevity regulating pathways were significantly downregulated (Fig. 3B). The DEPs in zooxanthellae that were used in the KEGG analysis are shown in Table 2.

The fructose and mannose metabolism pathway are related to polysaccharide biosynthesis (Zhou et al., 2020). The pentose phosphate pathway (PPP) is the main source of NADPH, especially in hypoxic environments (Carvalho et al., 2018). Changes in the expression levels of fructose-1,6-bisphosphatase (FBP) and fructose-bisphosphate aldolase (ALDO) were responsible for the changes observed in the fructose and mannose metabolism pathways and PPP. FBP is a key enzyme in mediating gluconeogenesis which catalyzes cleavage of fructose-1, 6-bisphosphate to fructose 6-phosphate and inorganic phosphate (Li et al., 2017). ALDO is a glycolytic enzyme that plays an important role in glycolysis and gluconeogenesis (Shen et al., 2020). FBP and ALDO were significantly increased from CM1 to TM2 and significantly decreased from TM2 to TM3. These results suggest that BaP may cause impairment

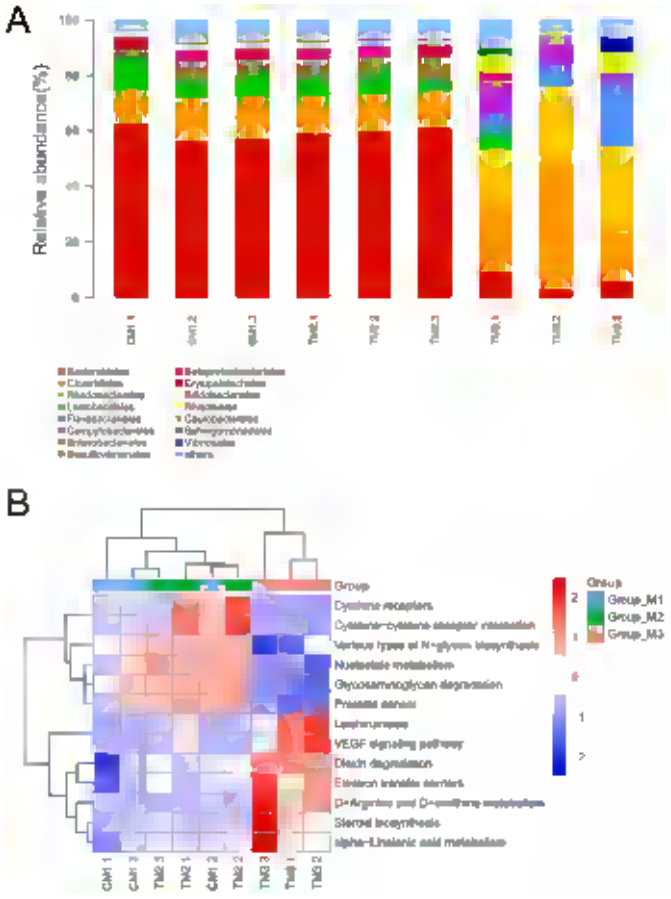

Fig. 4. Effects of BaP on the community and function of coral symbiotic microorganisms. (A) Displaying relative abundance changes of coral symbiotic microorganisms under BaP stress at the at the order level. (B) PICRUSt function prediction of coral symbiotic microorganisms under BaP stress.

of zooxanthellae sugar and oxygen synthesis, inducing key enzymes in gluconeogenesis and glycolysis to be upregulated.

The *FoxO* signalling pathway is closely related to apoptosis, cell cycle control, DNA repair, oxidative stress resistance, and longevity (Jalgaonkar et al., 2022). The MAPK signalling pathway is closely related to cellular proliferation, differentiation, and apoptosis (Katata et al., 2020). Superoxide dismutase, Fe–Mn family (SOD2) was downregulated in zooxanthellae from coral treated with BaP and contributed to downregulation of the *FoxO* and MAPK signalling pathways. SOD2 is an antioxidant enzyme localized to the mitochondrial matrix that acts to scavenge locally generated free radicals (Sharma et al., 2020). In the *FoxO* signalling pathway, SOD2 downregulation decreases oxidative stress resistance and DNA repair capacity in zooxanthellae; whereas, in the MAPK signalling pathway, SOD2 affects zooxanthellae longevity. These results suggest that BaP inhibits SOD2, reduces the oxidative stress resistance and DNA repair capacity of zooxanthellae, causes the accumulation of oxidative free radicals, and negatively affects zooxanthellae longevity.

Longevity regulatory pathways are associated with longevity in animals. Downregulation of the molecular chaperone DnaK (mtHSP70) is crucial for downregulation of the longevity regulatory pathway. MtHSP70 is also critical for mitochondrial protein biogenesis and plays a role in mitochondrial protein precursor import and folding (Shin et al., 2021). MtHSP70 is important for maintaining mitochondrial protein homeostasis, and a reduction in mtHSP70 leads to increased apoptosis via reduced mitochondrial membrane potential (Beider et al., 2020). The results indicate that decreased zooxanthellae mtHSP70 expression in the TM3 group may trigger zooxanthellae apoptosis.

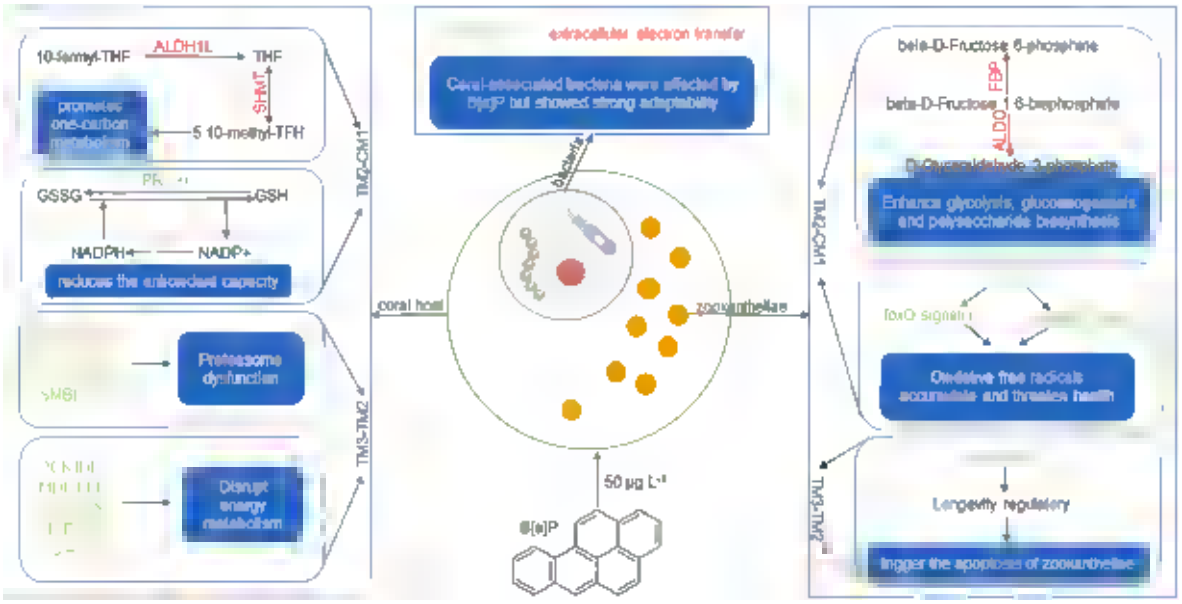

Fig. 5. The perturbation mechanism of BaP on the symbionts of *Montipora digitata*. Red means up regulation, green means down regulation. Abbreviations: ALDH1L: Formyltetrahydrofolate Dehydrogenase; THF: Tetrahydrofolate; 10-formyl-THF: 10-Formyltetrahydrofolate; SHMT: Serine Hydroxymethyltransferase; GSH: Glutathione; GSSG: Glutathione Disulfide; PRDX6: Peroxiredoxin 6; G6PD:Glucose-6-Phosphate1-Dehydrogenase; RPN1: 26S Proteasome Regulatory Subunit N1; RPT1: 26S Proteasome Regulatory Subunit T1; PSMA2: 20S Proteasome Subunit α2; PSMB4 : 20S Proteasome Subunit β4; MDH1: Malate Dehydrogenase; PCK: Phosphoenolpyruvate Carboxykinase; FH: Fumarate Hydratase; CS: Citrate Synthase; ACLY: ATP Citrate (pro-S)-Lyase; DLD: Dihydrolipoamide Dehydrogenase; PDHA: Pyruvate Dehydrogenase E1 Component Alpha Subunit; PC: Pyruvate Carboxylase; ACO: Aconitate Hydratase; IDH: Isocitrate Dehydrogenase; FBP: Fructose-1,6-Bisphosphatase; ALDO: Fructose-Bisphosphate Aldolase; SOD: Superoxide Dismutase; HSP70: Heat Shock Protein 70. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

In conclusion, from CM1 to TM2, BaP alters SOD2 expression, which may weaken the antioxidative stress and DNA repair abilities of zooxanthellae and cause an accumulation of oxidative free radicals. At the same time, upregulation of FBP and ALDO may enhance gluconeogenesis and glycolysis in zooxanthellae. From TM2 to TM3, BaP decreased FBP and ALDO protein expression, which may result in gluconeogenesis and glycolysis dysfunction in zooxanthellae, and concomitant decreases in mtHSP70-related protein expression may cause zooxanthellae apoptosis.

3.4. Perturbation effects of BaP on coral symbiotic microorganisms

Coral symbiotic microorganisms play an important role in the health of corals and the stability of the coral reef ecosystem (Li et al., 2022). Coral symbiotic microorganisms are highly dynamic, responding to changes in the coral host and external environment (Zhang et al., 2021). The results of *16S rRNA* amplicon pyrosequencing and analysis showed that BaP alters the relative abundance and function of coral symbiotic microorganisms.

The relative abundance of coral symbiotic microorganisms was analysed at the order level (Fig. 4A). The microbial community structures of CM1 and TM2 were similar, but a difference was observed in TM3. Compared with that of CM1 and TM2, the mean relative abundance of *Bacteria* in TM3 decreased from 60% to 6%, whereas *Rhodobacterales* increased from 0.3% to 26%. *Bacteroidales* are the dominant bacterial species in organisms and are closely related to energy metabolism and immunity (Gong et al., 2020b; Li et al., 2021c). *Rhodobacterales* are a widely distributed, abundant, metabolically versatile bacterial group found in the world's oceans (Isaac et al., 2021). Rhodobacterales are associated with various coral diseases, and they do not cause coral diseases directly, but are more likely to infest diseased corals (Pollock et al., 2017). For example, when corals were inoculated with *Vibrio coralliilyticus*, the relative abundance of *Vibrionales*, which was initially high, decreased and *Rhodobacterales* increased (Welsh et al., 2017).

A functional prediction analysis of coral symbiotic microorganisms showed that CM1 and TM2 bacteria had similar functions, and TM3 had obvious functional differences (Fig. 4B). Compared with those of CM1 and TM2, nucleotide metabolism and glycosaminoglycan degradation pathways were significantly downregulated in TM3, while steroid biosynthesis, electron transfer carriers, D-arginine and D-ornithine metabolism, and alpha-linolenic acid metabolism pathways were significantly upregulated. Downregulation of nucleotide metabolism and upregulation of steroid biosynthesis, D-arginine, D-ornithine, and alpha-linolenic acid metabolism indicate that BaP interferes with the energy metabolism pathways of coral symbiotic microorganisms. Glycosaminoglycan has multiple roles in immunity, and deficiencies in glycosaminoglycan degradation can cause lysosomal storage disorders and impair health (Kowalewski et al., 2012; Huo et al., 2020). The upregulation of electron transfer carriers can enhance the electron transfer ability of coral symbiotic microorganisms. Coral symbiotic microorganisms use extracellular electron transfer to exchange information and energy with other microorganisms and the external environment (Xiao et al., 2017).

In conclusion, BaP had no obvious disturbance effect on coral symbiotic microorganisms in CM1 and TM2, but there have significant changes of the relative abundance and function of microorganisms in TM3. BaP can cause the decrease of the immunity related coral symbiotic microorganisms, however, which exhibit some adaptive capacity to BaP stress by adjusting energy metabolic pathways and enhancing extracellular electron transfer capabilities.

4. Conclusion

Specific combinations of coral polyps, zooxanthellae, and coral symbiotic microorganisms form the so-called coral holobiont. Investigation of the mechanisms by which adverse factors affect the main coral symbionts (coral polyps, zooxanthellae, and coral symbiotic microorganisms) is critical for a comprehensive assessment of coral health. In this study, the diaPASEF proteomics and *16S rRNA* amplicon

pyrosequencing were used to explore the disruption effects of BaP on coral polps, zooxanthellae, and coral symbiotic microorganisms. The results showed that BaP at a concentration of 50 μg/L threatened the normal function and survival of *M. digitata*. Data collected from the symbionts at 0–72 h (CM1-TM2) of BaP treatment showed few differences, which indicated that *M. digitata* is not greatly affected by BaP at this stage. At 72–120 h (TM2-TM3) of BaP treatment, data collected from the symbionts changed greatly, indicating that the continuous stress induced by BaP may cause serious harm to *M. digitata* symbionts. In coral polyps, BaP impaired cellular antioxidant capacity, caused proteasome dysfunction and disrupted the TCA cycle; while in zooxanthellae, BaP inhibited the expression of antioxidant enzymes, caused an accumulation of oxidative free radicals and ultimately initiated apoptosis. In coral symbiotic microorganisms, BaP altered the bacterial communities, resulting in decreased immunity and bacterial adaptation by alterations in energy metabolism strategies and enhanced extracellular electron transfer (Fig. 5).

Author contributions statement

Yuebin Pei: Investigation, Methodology, Software, Data curation, Validation, Writing - original draft. **Shuai Chen**: Investigation, Validation, Software, Data curation. **Xiaoping Diao**: Investigation, Validation. **Xiaobing Wang**: Investigation, Validation. **Hailong Zhou**: Conceptualization, Writing -review & editing, Resources, Supervision. **Yuanchao Li:** Investigation, Validation. **Zhiyong Li:** Writing -review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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